Molecular And Genetic Analysis Of Signal Transduction Pathways Underlying *PR-1* Expression In *Arabidopsis*

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Doctor of Philosophy

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September 2001

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Declaration

I hereby declare that the work presented here is substantially my own and has not been submitted in any form for any degree at this or any other university.

Marjorie Santamaria

Publication derived from this work

Santamaria, M., Thomson, C.J., Read, N.D. and Loake, G.J. (2001). The promoter of a basic *PR1*-like gene, *AtPRB1*, from Arabidopsis establishes an organ-specific expression pattern and responsiveness to ethylene and methyl-jasmonate. *Plant Molecular Biology, 47*, 641-652..

Acknowledgements

I would like to thank, first of all, my supervisor, Dr. Gary Loake. Without his continued support and his talent, this work would not have been possible. I am also grateful to Prof. Kenneth Murray for giving me the opportunity to study at the University of Edinburgh. Thanks to all my colleagues in the lab for good advice and good laughs. Especially to Lena for her hard work on the mutant screen and her patience and good humour. Finally, thanks to my family and friends for making these four years in Edinburgh a brilliant experience.

Abstract

Antimicrobial proteins are a key feature underlying the deployment of both pre-formed and inducible defence responses. Probably the most well characterised class are the pathogenesis related (PR)-proteins, which are found in both basic and acidic isoforms. This study describes the isolation and characterisation of a gene, designated *AtPRB1*, encoding a basic PR1-like protein from *Arabidopsis*. This protein showed high amino acid sequence identity with basic and acidic PR1 proteins from other plant species for example, PRB1 from *Nicotiana tabacum* and PR1 from *Brassica napus*, at 64% and 78% identity respectively. A genomic DNA fragment containing 2345 bp upstream from the putative transcriptional start site was fused to the gene encoding the luciferase (*LUC*) gene, in order to test for promoter activity. The resulting construct was transformed into *Arabidopsis* accession Col-0 and analysis of LUC activity, using an ultra low light imaging camera system, revealed that the *AtPRB1* promoter established an exquisite organ-specific expression pattern. LUC activity was observed in flowers, stems and roots but not in leaf tissue. Superimposed upon this organ-specific expression pattern was responsiveness, in root tissue, to ethylene (ET) and methyl-jasmonate (Me-JA), important cues during the establishment of plant disease resistance. In contrast, *AtPRB1*::*LUC* gene expression was repressed in response to salicylic acid (SA) treatment. Analysis of a limited series of *AtPRB1* 5′-promoter deletion mutants, identified a number of promoter regions important for both the establishment of organ-specific expression and responsiveness to ET and Me-JA. While *AtPRB1* gene expression was not induced in response to an avirulent isolate of *Peronospora parasitica* in leaf tissue, this gene may contribute to constitutive resistance in other tissues and/or to Me-JA and ET dependent defence responses engaged against necrotrophic pathogens in root tissue.

In the second part of this study, a population of 5000 activation-tagged lines was generated in a *PR-1*::*LUC* genetic background to uncover novel systemic acquired resistance (SAR) mutants. The mutant screen involved imaging for constitutive LUC expression or absence of LUC expression after induction with the SA functional analogue, BTH. A set of five mutants displaying constitutive LUC activity were selected for further characterisation. Four mutants, designated *esr2*, *esr3*, *esr5* and *esr6* for *e*nhanced *s*ystemic *r*esistance, exhibited constitutive *PR-1* gene expression and increased resistance to the virulent oomycete pathogen *P.parasitica* Noco2. Analysis of crosses between the lesion mimic mutant *esr2* and other mutants that disrupt SA and JA/ET signalling showed that *PR-1* expression in *esr2* was largely but not strictly dependent on SA signalling. Furthermore, the ET- and JA-insensitive *etr1* and *coi1* mutants were able to block *PR-1* expression when introduced into *esr2*. Based on these results, ESR2 could act as a regulator of a signalling pathway that requires components of the SA, and JA/ET pathways. Further characterisation and cloning of *esr* mutants, should provide valuable insights into the function, structure and molecular interactions of novel genes involved in disease resistance.

Abbreviations

µg	microgram
µl	microlitre
ABA	abcissic acid
bp	base pair
BTH	benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
CaMV	Cauliflower Mosaic Virus
cfu	colony forming units
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
ET	ethylene
g	gram
JA	jasmonate
KB	King's broth media
kD	kilodalton
LUC	luciferase
Me-JA	methyl-jasmonate
mg	milligram
ml	millilitre
MS	Murashige and Skoog media
MW	Molecular weight standard
nahG	salicylate hydroxylase gene
PCR	polymerase chain reaction
PR	pathogenesis-related
prot	protein
Pst	*Pseudomonas syringae* pv *tomato*
r18	ribosomal 18S
RNA	ribonucleic acid
RLU	Relative Light Units
T-DNA	transfer DNA

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CHAPTER 1

Introduction

1.1 General Introduction

Despite the use of fungicides and breeding strategies to produce resistant varieties, plant disease is still causing serious crop losses, estimated to be 13% of global potential agricultural production (James *et al.*, 2001). The use of high-yield monoculture crops with little genetic diversity, results in enhanced susceptibility to increasingly aggressive pathogens, leading to intensive chemical control which is both costly and harmful to the environment. With population growth, expansion of farming areas will also be necessary, putting additional pressure on rain forests and wilderness areas. Consequently, the research effort has been directed towards developing novel methods for controlling plant diseases and increasing crop yield. Recent improvements in plant transformation techniques and progress in the understanding of plant-pathogen interactions enable the use of genetic engineering for the creation of disease resistant plants. Different genetic strategies have been proposed to engineer resistance, including expression of insecticidal, anti-bacterial and anti-fungal proteins of non-plant origin, inhibition of pathogenicity or virulence factors, induction of programmed cell death at the site of infection and enhancement of plant innate defences. While the introgression of resistance genes from wild species has proved useful, resistance mediated via this mechanism is generally not durable. Mutations in the pathogen avirulence (*avr*) genes are known to occur frequently and therefore resistance mediated by the introgression of a single resistance (*R*) gene can be rapidly overcome. It is likely that combined strategies on a given plant genotype will decrease the risk of resistance development in the pathogen.

Genetically modified crops were first planted on a commercial scale in the mid nineties. At present, more than half of the world's soybean and about one third of the corn crop is transgenic (James, 2000). Nevertheless, the use of the *Bacillus*

thuringensis insecticidal toxin gene in corn and other transgenes in plants is subject to increasing concern, due to their potential toxicity or allergenic risks to humans or animals when transgenic plants are consumed. Moreover, there is also the risk of cross-pollination with related species and unintended environmental effects. It is therefore of great interest to understand the genetic and molecular basis of plant disease resistance because it may contribute to the development of transgenic crops with increased endogenous protection against pathogens. *Arabidopsis thaliana* has proved extremely useful as a model for plant pathogen interactions. Its small size, rapid generation time (6-10 weeks), small genome size (130 Mb) and low repetitive DNA content has facilitated the identification and mapping of over 49 resistance specificity loci (Buell *et al.*, 1998). The information afforded by its genome sequence and detailed genetic map provides the basis to investigate the signal transduction pathways leading to resistance. Sequence conservation of *R* genes and signalling components among plant species suggests that findings will be relevant to important crop species.

1.2 Gene-for-gene resistance

Plants have developed elaborate defensive strategies to prevent pathogen infection. Resistance can result from the existence of preformed structural and chemical barriers. The presence of thick, wax cuticles (Jackson and Taylor, 1996) and antimicrobial secondary metabolites such as saponins (Osbourn, 1996), have been implicated in resistance. However, plants also possess inducible defence mechanisms. These inducible responses are often activated by the direct or indirect interaction of a host *R* gene product and a pathogen *avr* gene product as proposed by the gene-for-gene hypothesis (Flor, 1971). The absence of any one of the two gene products leads to disease susceptibility. A simple explanation for the molecular basis of gene-for-gene interactions is a receptor-ligand model where the plant resistance gene acts as a receptor that recognises the products of avirulence genes in the pathogen. Recognition triggers both a programmed cell death response, known as the hypersensitive response (HR) around the site of infection and the release of a

systemic signal that activates systemic acquired resistance (SAR) throughout the plant (Ryals *et al.*, 1996). Alternatively, the pathogen may lack the corresponding *avr* gene to interact with an *R* gene, in which case the host plant will fail to induce its defence responses. Plants that are susceptible to virulent pathogens still deploy defence responses. Many of the defence responses observed in resistant plants, with the exception of the HR are also activated in susceptible plants, although usually more slowly and weakly (Van Loon, 1997). Defence responses that have been observed after infection by either virulent or avirulent pathogens include the strengthening of cell walls by lignification, suberization, callose deposition, synthesis of phytoalexins and induction of the so-called pathogenesis related (PR) proteins (Yang *et al.*, 1997).

1.3 Resistance-genes

Molecular characterisation of several complementary pairs of plant *R* genes and pathogen *avr* genes has made it possible to test the gene-for-gene receptor-ligand concept (Fig. 1.1). Direct interaction between *R* and *avr* gene products in the yeast two-hybrid system has only been demonstrated for the tomato Pto and *Pseudomonas* AvrPto proteins (Scofield *et al.*, 1996). *Pto* is a unique *R* gene which encodes a serine-threonine kinase that confers resistance to bacterial speck disease. Several lines of evidence suggest a specific binding event which is essential for activation of the disease resistance pathway (Tang *et al.*, 1996). Firstly, an active Pto kinase from a bacterial speck susceptible cultivar failed to bind avrPto in the yeast two-hybrid system. Secondly, a single amino acid residue, Thr204 of the Pto kinase, determined the recognition specificity. Thirdly, C-terminal fragments of the AvrPto protein that interact with Pto were capable of eliciting HR and disease resistance in plants (Tang *et al.*, 1996).

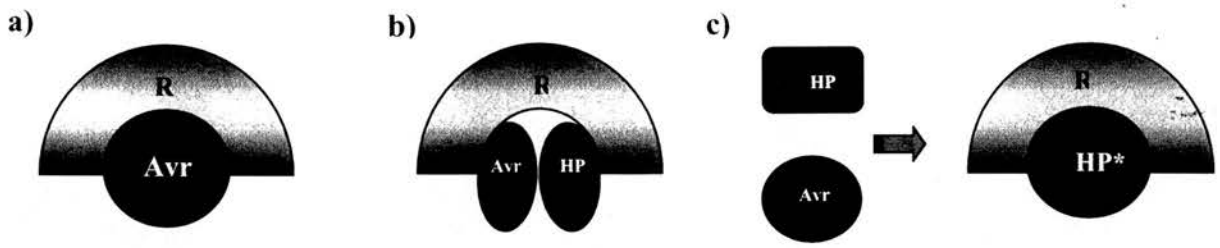

Figure 1.1. Three versions of the receptor-ligand model for the direct/indirect interaction of R and Avr proteins. a) This model is consistent with plant-virus interactions. Recognition of a particular Avr protein structure triggers the resistance response. **b)** The second model assumes that the interaction of the Avr protein with one or more host proteins (HP) leads to recognition by the R receptor. **c)** In the third model the R receptor may not directly interact with any pathogen determinant but may indirectly recognise the product of Avr-HP activity through the conformational change imposed on the host protein (HP*). The Pto-AvrPto interaction might be an example of this situation (see text, *R*-gene section). Binding of AvrPto to Pto in wild type plants may lead to a conformational change and activation of Pto kinase, thereby providing a critical conformational substrate for the Prf-triggered HR response (based on Ellis *et al.*, 2000).

The numerous *R* genes that have been cloned from different plant species in the last few years seem to share structural features such as leucine rich repeat motifs (LRR), protein kinase domains and nucleotide binding sequences (NBS) (Staskawicz *et al.*, 1995). These similarities suggest that common signalling events occur in response to diverse pathogens. Leucine rich repeats have been implicated in protein-protein interactions and ligand binding (Jones, 1996). Presumably, LRRs are receptor domains for Avr proteins and sequence variation within the repeats determines recognitional specificity (Hu *et al.*, 1996; Jones and Jones, 1997). In support of this idea, the exposed residues of a parallel ß sheet composed of leucine repeats from a porcine ribonuclease inhibitor protein were found to form a surface for ligand interactions (Kobe and Deisenhofer, 1995). In several *R* gene proteins, the predicted exposed residues in the ß sheet regions are highly variable and correlate with differential pathogen recognition (Noel *et al.*, 1999). *R* genes have been divided into

NBS/LRR gene, *Prf,* is required for resistance encoded by *Pto* (Martin *et al.*, 1993). Because both *Pto* and *Prf* are essential for resistance, this implicates LRR-containing proteins and protein kinases in the same signalling pathway. The second class of *R* genes encode transmembrane receptors with extracellular LRR domains and is represented by the *Cf* family in tomato, specific for *Cladosporium fulvum* resistance (Hammond-Kosack and Jones, 1997). The third class combines features of both the above classes and is exemplified by *Xa21* which confers resistance to rice bacterial blight. *Xa21* encodes a receptor-like kinase and provided the first indication of the link between a receptor function in *R* proteins and potential downstream signalling capacity (Song *et al.*, 1995). The fourth class includes the majority of *R* genes described so far, which contain three common domains: a variable N-terminus, nucleotide binding site and leucine rich repeats (Bent, 1996). Two types of N termini are present in NBS-LRR resistance genes and therefore this class has been subdivided in two subclasses. One subclass contains coiled-coils (CC) that are thought to have a role in protein-protein interactions (Lupas, 1996). The other subclass has been described only in dicots and shows homology to the drosophila Toll and human interleukin receptor-like (TIR) regions (Baker *et al.*, 1997). The fifth class includes a unique type of resistance gene. Unlike other characterized R genes, RPW8.1 and RPW8.2 conferred resistance to a wide range of powdery mildew diseases of *Arabidopsis*, reminiscent of that conferred by recessive alleles at the barley MLO locus (Xiao *et al.*, 2001).

R genes are often clustered on particular chromosomes and these regions may be highly variable. The possibility of generating new *R*-gene specificities by sequence exchange events such as interallelic recombination and gene conversion has been proposed in studies of the flax-rust system which contains 30 or more alternative alleles specifying resistance to flax rust (Dodds *et al.*, 2001). However, a direct recombination event has not yet been observed.

1.4 *Avr* genes

In contrast to the similarities among cloned plant *R* genes, sequence analysis of cloned *avr* genes revealed few similarities and few clues to their function in the pathogen (Dangl, 1994). Although their biochemical activities or virulence functions remain unclear, several Avr proteins are known to make measurable contributions to virulence (Leach and White, 1996). For instance, members of the AvrBs3 family in *Xanthomonas* spp. are targeted to the plant cell nucleus and some of these redundantly produce water soaking symptoms associated with virulence (Yang *et al.*, 1996). Since mutations in some *avr* genes do not detectably reduce pathogen virulence, it is suspected that they have overlapping, complementary functions with other virulence factors (Ji *et al.*, 1998).

Many bacterial Avr proteins, including AvrRpt2 and AvrB of *Pseudomonas syringae,* commonly used as a pathosystem in *Arabidopsis* studies, are believed to be transferred directly into the plant cell (Kjemtrup *et al.*, 2000). The *R*-gene surveillance system may then recognise the Avr protein in the cell cytoplasm. Two lines of indirect evidence support this notion. First, most *avr* genes require the hypersensitive response and pathogenicity (*hrp*) gene cluster, which encodes components of the bacterial type III secretion system, for effective *avr* delivery and functioning (Alfano and Collmer, 1996). Second, the expression of *avr* genes directly in plant cells is sufficient to trigger an *R*-gene mediated defence response (Gopalan *et al.*, 1996; Scofield *et al.*, 1996).

The cellular location of *R* genes is thought to reflect the cellular location of *avr* gene products. Hence, those *R* genes that predominantly respond to fungal pathogens carry an extracellular LRR region and a cytoplasmic signalling domain (Dixon *et al.*, 1996). The avirulence products from fungal pathogens are presumed to bind the receptor in the extracellular space (Thomas *et al.*, 1997). In the case of *Peronospora parasitica*, an oomycete parasite that forms feeding structures inside the plant cell, Avr proteins may be delivered intracellularly. Their corresponding R proteins are

predicted to be intracellular (McDowell *et al.*, 1998). In bacterial plant pathogens, a specialised (Type III) protein secretion apparatus mediates transfer of bacterial proteins into the cytoplasm of the host cell where they are most likely recognised by the corresponding plant *R* gene product (Alfano and Collmer, 1997; Bonas and Van den Ackerveken, 1997).

1.5 Mutants in *R*-gene mediated signalling

R genes function at or near the beginning of a complex signal transduction cascade that leads to HR and ultimately to SAR. Mutant screens in *Arabidopsis* revealed two mutants, *ndr1* for *n*on-race-specific *d*isease *r*esistance (Century *et al.*, 1995) and *eds1* for *e*nhanced *d*isease *s*usceptibility (Parker *et al.*, 1996), displaying altered *R*-gene dependent responses. Several *R*-genes (*Rpp2, Rpp4*, *Rpp5*, *Rpp21*, *Rps4*) were shown to require EDS1 for resistance to *P.parasitica* and *P.syringae* (Aarts *et al.*, 1998). Surprisingly, resistance mediated by these genes was unaffected or only weakly affected by the *ndr1* mutation. In contrast, a different set of *R*-genes (*Rps2*, *Rpm1*, *Rps5*) was shown to require NDR1 but not EDS1 (Aarts *et al.*, 1998). Based on these results, NDR1 and EDS1 define convergence points of at least two different signalling pathways. The structure of the R protein seems to determine which pathway is activated. The EDS1-dependent *R*-genes belong to the TIR-NBS/LRR class whereas NDR1-dependent *R*-genes belong to the CC-NBS/LRR class. Interestingly, Rpp8 which does not strongly require either EDS1 or NDR1, is a CC-NBS/LRR type of protein (McDowell *et al.*, 2000). Therefore, more than just the amino-terminal domain defines the signalling pathway. The EDS1 gene encodes a protein with homology to eukaryotic lipases (Falk *et al.*, 1999). It is then possible for EDS1 to regulate defence responses by processing a lipid-derived signal. NDR1 encodes a transmembrane protein with unknown function (Century *et al.*, 1997).

1.6 The Hypersensitive Response

Following perception of an Avr protein from the pathogen by an *R* gene product in the plant, a signal transduction cascade is rapidly activated that leads to the development of hypersensitive cell death (HR) (Hammond-Kosack and Jones, 1996). The HR is characterised by the synthesis of phytoalexins (Dixon, 1986), the reinforcement of cell walls by lignin polymerisation and suberisation (Vance *et al.*, 1980), the rapid generation of reactive oxygen intermediates (ROIs)(Doke, 1983; reviewed by Grant and Loake, 2000), induction of PR-proteins (Bol *et al.*, 1990) and hypersensitive cell death of challenged cells (Morel and Dangl, 1997), which together prevent further spread of the disease. Pathogen containment could result from nutrient deprivation or direct exposure to toxic compounds, or both.

1.7 Mutants that show altered control of HR cell death

Evidence that HR cell death is a form of programmed cell death controlled by the plant derives from the existence of mutants, called "lesion-mimics" that exhibit spontaneous appearance of necrotic lesions in the absence of pathogen. In *Arabidopsis, a*ctivated *c*ell *d*eath (*acd*) mutants (Greenberg and Ausubel, 1993; Greenberg *et al.*, 1994; Rate *et al.*, 1999) and *l*esion *s*imulating *d*isease resistance (*lsd*) mutants (Dietrich *et al.*, 1994; Weymann *et al.*, 1995) have been identified. In these mutants, activation of SAR is correlated to the development of lesions. On the basis of their phenotypes, two classes of lesion-mimic mutations were defined (Morel and Dangl, 1999). In the first class, lesions are confined and it was hypothesised that these mutations represent defects in genes involved in triggering the HR pathway (Dangl *et al.*, 1996; Mittler and Lam, 1996). In the second class of mutations, lesions spread once they have been initiated until the entire leaf eventually becomes necrotic (Dietrich *et al.*, 1994). These mutations define genes necessary to control the extent of cell death (Dietrich *et al.*, 1997). The *dnd1* (*d*efense, *n*o *d*eath) mutant can limit pathogen growth, constitutively accumulates SA

and mounts a SAR response (Yu *et al.*, 1998). However, unlike other constitutive SAR mutants (see below), *dnd1* does not develop HR lesions following infection with avirulent *P.syringae*. These findings demonstrate that HR cell death can be uncoupled from gene-for-gene resistance in *Arabidopsis*. DND1 has been recently cloned and is predicted to encode a cyclic nucleotide-gated ion channel (Clough *et al.*, 2000).

While there is evidence that cell death is not required for defence gene induction and in many cases it is not essential for a resistance response, there is a strong correlation between host cell death and the effectiveness of the resistance response. For example, in the *Arabidopsis cim* mutants which show increased resistance to pathogens but no HR cell death, resistance is quantitative in nature, with partial but not total restriction of pathogen (Frye and Innes, 1998). Moreover, analysis of a large number of barley *mlo* alleles has established a positive correlation between the frequency of necrosis and the effectiveness of resistance (Buschges *et al.*, 1997).

1.8 Systemic Acquired Resistance

HR and other local necrotic reactions are believed to trigger non-specific resistance throughout the plant associated with SAR (Ward *et al.*, 1991). This resistance response, first characterised by Ross (1961), is expressed systemically and is effective against secondary infections by a broad spectrum of viral, bacterial and fungal pathogens. Enhanced resistance can last from several days in dicots to several months in monocots (Gorlach *et al.*, 1996). The establishment of SAR is preceded by an increase in salicylic acid (SA) and it is correlated with induction of the PR-proteins. Activation of PR expression therefore has been used as a convenient marker of SAR. In addition, both *in vitro* and *in vivo* studies have demonstrated that many PR-proteins are effective anti-microbial agents. However, overexpression of PR-proteins does not always confer enhanced resistance to pathogen attack (Neuhaus *et al.*, 1991). Thus, SAR clearly involves numerous other components that have yet to be identified.

1.8.1 The role of reactive oxygen intermediates and nitric oxide

The earliest detectable cellular events following pathogen infection are ion fluxes across the plasma membrane and an oxidative burst that produces ROIs, predominantly superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Doke *et al.*, 1988; Low and Merida, 1996). The oxidative burst takes place shortly after attempted infection in susceptible and resistant interactions. But a second, sustained burst occurring within a few hours, is correlated to resistance (Levine *et al.*, 1994). A number of possible roles for ROIs have been proposed including direct toxicity on pathogens (Peng and Kuc, 1992), strengthening of cell walls by oxidative cross-linking of structural proteins (Bradley *et al.*, 1992), promotion of programmed cell death (PCD) during the HR and induction of defence gene expression (Lamb and Dixon, 1997). The identification of plant homologs of mammalian NADPH oxidase and two NADPH oxidase components (ap91phox and Rac) involved in ROI production in neutrophils pointed to the potential function of plant ROIs in disease resistance (reviewed by Bolwell, 1999). In plants, activation of a cell surface NADPH oxidase results in local synthesis of superoxide which spontaneously dismutates to hydrogen peroxide. A requirement for NADPH oxidase in plant cell death is further supported by observations that pharmacological inhibitors of the NADPH oxidase can interfere with induction of the HR (Lamb and Dixon, 1997). However, alternative sources of ROIs have been described but their relative contribution is controversial (Grant and Loake, 2000).

There is strong evidence that PCD is separable from ROI production. A number of cell elicitation systems showed that exogenously supplied ROIs are insufficient to activate HR cell death (Richberg *et al.*, 1998; Piedras *et al.*, 1998). Therefore, ROIs are necessary but not sufficient to trigger cell death. Two other possible players have been identified: salicylic acid and nitric oxide (NO). NO seems to be the most important component as it was capable of inducing HR in soybean cells when supplied together with ROIs (Delledone *et al.*, 1998). Furthermore, exogenous application of NO lead to SA accumulation and increased levels of PR-gene

expression (Durner *et al.*, 1998). Expression of these genes was also induced by cyclic GMP and cyclic ADP ribose, two molecules that can act as second messengers for NO signalling in animal cells (Durner *et al.*, 1998). ROIs may function in combination with NO, possibly after reacting together to form highly toxic peroxynitrite and hydroxyl radicals (Dangl, 1998).

Interaction of NO with ROIs and SA to induce the HR and defence gene expression has also been documented. Evidence that SA is involved in the induction of cell death, possibly via a feedback loop, has come from studies on soybean cultured cells in which pre-exposure to SA can accelerate cell death upon challenge with an avirulent pathogen (Shirasu *et al.*, 1997). Following the activation of the oxidative burst is the rapid expression of phenylalanine ammonia lyase (PAL) which presumably catalyses the first step in the synthesis of SA. This observation suggests that SA synthesis occurs at an early point, before the second phase of sustained ROI production induced in response to avirulent pathogens (Draper, 1997). In support of this idea is the demonstration that a PAL inhibitor can greatly reduce both H_2O_2 synthesis and cell death induced by avirulent bacteria. The effect of the inhibitor could be suppressed by the addition of SA (Mauch-Mani and Slusarenko, 1996). In this system, SA in combination with cantharidin, a protein phosphatase inhibitor, is able to activate ROI production in the absence of pathogen. In conclusion, the potentiation effect of SA occurs through an undefined phosphorylation-dependent agonist that regulates ROI production. The oxidative burst, in turn may drive further SA production (Leon *et al.*, 1995), resulting in a positive feedback cycle. These features indicate that *R*-gene-dependent pathogen perception triggers a positive feedback loop of ROI, NO and SA accumulation, which rapidly amplifies the initial signal and culminates in HR (McDowell and Dangl, 2000).

ROIs accumulated during the HR have recently been implicated in SAR activation. Establishment of SAR following avirulent *Pst* inoculation was correlated with induction of systemic micro-lesions in periveinal cells from uninfected tissues (Alvarez *et al.*, 1998). The implication is that systemic resistance can be induced by

micro-HRs throughout the plant. However, the short half-life of ROIs suggests they are not the actual "mobile signal" that induces micro-HRs and SAR.

1.8.2 The role of salicylic acid

SA has long been known to play a key function in signalling for disease resistance. Exogenous SA is able to induce plant defence gene expression and SAR (Uknes *et al.*, 1992; Vernooij *et al.*, 1995). Definitive proof that SA is required for SAR was provided by experiments with transgenic tobacco and *Arabidopsis* plants expressing a bacterial salicylate hydroxylase gene (*nahG*). This enzyme catalyses the conversion of SA to inactive cathecol. Transgenic *nahG* plants fail to accumulate SA, do not establish SAR (Gaffney *et al.* 1993; Delaney *et al.*, 1994) and show increased susceptibility to avirulent and virulent *Pseudomonas syringae* pv. tomato (DC3000), *Peronospora parasitica* and *Erysiphe orontii* (Delaney *et al.* 1994; Lawton *et al.* 1995; Reuber *et al.*, 1998). Moreover, in *Arabidopsis* resistance to infection by *P. parasitica* was completely abolished by treatment with a PAL inhibitor (Mauch-Mani and Slusarenko, 1996). Treating *nahG* expressing plants with SA, and 2,6-dichloroisonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), two functional analogues of SA, restored SAR and *PR-1* expression to wild-type levels (Lawton *et al.*, 1996; Vernooij *et al.*, 1995). SA induction-deficient (*sid*) mutants of *Arabidopsis* also show decreased expression of a key SAR gene and increased susceptibility to microbial pathogens (Nawrath *et al.*, 1999). Conversely, increasing SA concentration by endogenous synthesis was sufficient for SAR establishment (Mauch *et al.*, 2001).

The involvement of SA in local resistance was shown with crosses between the dominant mutants *lsd6* and *lsd7* to SA depleted *nahG* plants (Weymann *et al.*, 1995). In the resulting F_1 plants, lesion formation was suppressed and in the case of *lsd6xnahG* it was restored by exogenous application of SA. One possible explanation is the positive feedback loop that exacerbates lesion formation mediated by SA (Weymann *et al.*, 1995). In contrast, when *lsd2, lsd4* and *lsd5* mutants were crossed

to *nahG* plants, the lesion phenotype was not suppressed but SAR and *PR*-gene expression were eliminated (Hunt *et al.*, 1997). Surprisingly, *nahG* does not suppress the spreading lesion phenotype in *lsd1* plants, indicating that SA is not required for lesion initiation (Dietrich *et al.*, 1994). Thus, lesion formation in *lsd1* can be uncoupled from resistance. Taken together, these results suggest the existence of SA-dependent and SA-independent cell death pathways.

SA has also been postulated to be the long-distance SAR signal that moves from the inoculated leaf to systemic leaves and activates defence responses. The rise in SA levels that precedes *PR* gene induction in uninoculated leaves of TMV infected tobacco indicated that SA might fulfill this function (Delaney *et al.*, 1994). Furthermore, a large increase in SA in the phloem from cucumber-infected leaves and radioactive-labelling experiments in which ^{14}C-labelled SA was found to be translocated to upper uninfected leaves, demonstrated that indeed, SA is mobile in the plant (Moelders *et al.*, 1996; Shulaev *et al.*, 1995). However, an increasing body of evidence suggests this hypothesis is incorrect. In *P.syringae*-infected cucumber, the SAR signal moved out of the inoculated leaf before an increase in SA level was detected in the petiole (Rasmussen *et al.*, 1991; Smith *et al.*, 1991). In addition, wild-type scions of chimeric tobacco plants were able to induce PR-1 expression and SAR following TMV-inoculation of *nahG* rootstock leaves (Vernooij *et al.*, 1994).

The current data supports the idea that SA functions at multiple points in the SAR signalling pathway (Weymann *et al.*, 1995), possibly by acting as a signal amplifier (Conrath *et al.*, 1995; Mur *et al.*, 1996; Shirasu *et al.*, 1997). Since SA accumulation is crucial for local resistance as well as systemic resistance, it is possible that SA and a second, unidentified signal are required to activate SAR (Dempsey *et al.*, 1999).

1.8.3 Pathogenesis-Related proteins

Originally, the PR-proteins were identified in tobacco leaves infected with TMV (Van Loon and Van Kammen, 1970) and grouped into five families, based on sequence homology. The system was revised by Van Loon and now includes 14 different families (Van Loon and Van Strien, 1999) (Table 1.1).

Table 1.1 Families of PR proteins (adapted from Hoffmann-Sommergruber 2000)

Family	Properties/designations	MW(kD)
PR-1	unknown	15-17
PR-2	antifungal/class I, II, IIβ-1,3-glucanases	25-35
PR-3	antifungal/class I, II, IV chitinases	25-35
PR-4	antifungal/class I, II chitinases	13-19
PR-5	antifungal/thaumatin-like proteins	22-24
PR-6	proteinase inhibitors	6
PR-7	endoproteinases	69
PR-8	class III chitinases/lysozymes	28
PR-9	peroxidase isoenzymes	39-40
PR-10	unknown/ Bet v 1-homologues	17-18
PR-11	class I chitinases	41-43
PR-12	defensins	5
PR-13	thionins	14
PR-14	lipid transfer proteins	7-12

The term PR comprises proteins with different biochemical and enzymatic activity, although in some cases the nature of this activity is not yet known. The unifying feature is that they are induced by pathogen attack either by abiotic factors (such as wounding) or biotic factors (such as pathogen infection). PR-proteins are usually small in size (ranging from approximately 5-70 kD), stable at low pH conditions and relatively resistant to proteolysis. These features are useful during disease and stress conditions, when the plant metabolism is trying to cope with extreme situations. PR-

proteins accumulate at the site of attempted infection and systemically during the onset of SAR in several plant species, including tobacco, *Arabidopsis*, tomato, potato and cucumber (Van Loon *et al.*, 1994). They are also found in maize, barley, wheat and rice (Casacuberta *et al.*, 1991; Muradov *et al.*, 1993; Morris *et al.*, 1998; Gorlach *et al.*, 1996). However, their contribution to SAR in monocots has not been established. The nature and level of expression of PR-proteins vary among plant species. In tobacco and *Arabidopsis*, acidic PR-1 is the main protein induced during SAR, whereas in cucumber PR-8 is induced to high levels but PR-1 is weakly expressed.

For the majority of the PR families, activities are known or can be inferred (Linthorst, 1991). The PR-2 family is composed of β-1,3-glucanases which catalyse the hydrolytic cleavage of the 1,3-β-glucosidic linkages in glucans, an abundant component of cell walls (Hoj *et al.*, 1995). They may be effective against fungal infection via enzymatic degradation of pathogen cell walls and release of cell wall-derived components that act as elicitors of defence responses (Bowles, 1990). They are also implicated in a number of physiological and developmental processes in healthy plants including pollen tube growth, fertilisation, fruit ripening, seed germination and mobilisation of storage products in the endosperm (Leubner-Metzger and Meins, 1999). Moreover, they are induced in response to wounding, cold stress and ozone stress by ethylene (Leubner-Metzger and Meins, 1999). The PR-3, -4, -8 and –11 families are all classified as endochitinases with basic isoforms exhibiting lysozyme activity. They are capable of hydrolysing chitin, the major component of the exoskeleton of insects as well as of the cell wall of fungi and nematodes (Collinge *et al.*, 1993). The seven distinct structural classes were suggested to represent complementary enzymes with synergistic effects on their substrates (Brunner *et al.*, 1998). Likewise, a combination of β-1,3-glucanase (PR-2) and chitinase (PR-3) demonstrated their synergistic effect against *Fusarium solani* (Melchers *et al.*, 1993). Like other PR-proteins, chitinases have been shown to be pathogen inducible and developmentally regulated in an organ-dependent manner (Lawton *et al.*, 1992; Samac *et al.*, 1990). Coordinate induction of acidic isoforms in response to biotrophic pathogens is SA-mediated. Expression of basic isoforms in

flowers, roots and lower leaves can be induced by pathogen challenge, wounding, heavy metals, abcissic acid (ABA) and is thought to be jasmonate (JA)/ethylene (ET)-mediated (Xu *et al.*, 1994). The PR-5 family has also been designated thaumatin-like proteins and they show homology to permatins, that permeabilise fungal membranes (Vigers *et al.*, 1991). PR-6 are proteinase inhibitors implicated in defence against insects and other herbivores and nematodes (Koiwa *et al.*, 1997). PR-7 has so far been characterised only in tomato, where it is a major PR and functions as an endoproteinase. It probably collaborates in cell wall lysis via cell wall protein degradation (Haran *et al.*, 1996). The PR-9 family of peroxidases is likely to function in cell wall strengthening by lignin deposition following pathogen attack (Van Loon *et al.*, 1994). The PR-10 family is structurally related to ribonucleases but their possible ability to cleave viral RNA remains to be demonstrated (Constable *et al.*, 1995). The PR-12 type defensins, PR-13 type thionins and PR-14 type lipid-transfer proteins all exhibit membrane destabilising activity and are associated with resistance to necrotrophic fungi (Broekaert *et al.*, 1997; Bohlmann, 1994; Garcia-Olmedo *et al.*, 1995).

The only PR family for which no biochemical function or relationship is known, consists of the PR-1 proteins. Direct antifungal activity of tomato PR-1 was shown *in vitro* as an inhibition of *Phytophtora infestans* spore germination, and *in vivo* as a reduction of the infected area of leaves (Niderman *et al.*, 1995).

The initial five PR families were all shown to have antifungal activity *in vitro* and consist of acidic as well as basic isoforms. Acidic PR-proteins are inducible by SA and characteristically secreted into the plant apoplast (Parent and Asselin, 1984). Basic isoforms occur at relatively low levels in the plant vacuole (Cutt and Klessig, 1992). The expression of basic PR-proteins can be triggered by the plant hormone ET and/or JA which are known to function as key signals in both the establishment of host resistance and disease symptom development (Thomma *et al.*, 1998; Lund *et al.*, 1998; Reymond and Farmer, 1998).

1.9 Salicylic acid-dependent signalling

While there has been considerable progress in understanding the events that take place during the establishment of SAR, little is known about the signal transduction network that underlies disease resistance. The isolation and characterisation of mutants defective in SA signalling has initiated the dissection of this signalling pathway.

1.9.1 NPR1-dependent signalling

From the SAR-compromised mutants, *npr1* (also known as *nim1/sai1*) is the only gene identified that affects a function downstream of the SA signal (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook *et al.*, 1996; Ryals *et al.*, 1997; Shah *et al.*, 1997). NPR1 is a positive regulator of defence responses. The *npr1* mutants are impared in the ability to induce *PR*-genes or to mount a SAR response in the presence of SA or SAR inducers such as INA or BTH. The NPR1 gene encodes a novel protein containing ankyrin repeats (Cao *et al.*, 1997; Ryals *et al.*, 1997), which are found in proteins of diverse functions, including IκB, which regulates animal immune responses (Lemaitre *et al.*, 1996). In a yeast two-hybrid screen, NPR1 was found to bind specifically to a subclass of bZIP transcription factors designated TGA and AHBP-1b factors (Zhang *et al.*, 1999). A screen for suppressors of *npr1* identified *sni1*, a leucine rich nuclear protein (Li *et al.*, 1999). In wild-type plants, SNI1 is a repressor of *PR*-genes and therefore, a negative regulator of SAR. Li *et al.* proposed a model in which induction of *PR*-gene expression and SAR requires both activation of a positive regulator presumably one or more TGA factors by SA and de-repression of SNI1 by the SA-activated NPR1. The mechanism by which NPR1 may inactivate SNI1 is not clear as no direct interaction between these two proteins was detected in the two-hybrid analysis.

1.9.2 Enhanced disease susceptibility mutants

At least thirteen mutants that displayed *e*nhanced *d*isease *s*usceptibility (*eds*) to virulent *P.syringae* pv *maculicola* have been identified (Glazebrook *et al.*, 1996; Rogers and Ausubel, 1997; Volko *et al.*, 1998). They provided evidence that the growth of virulent pathogens is actively limited by host defence responses. A subset of these mutants was also found to be more susceptible to infection by *E. orontii* showing that different defence responses are deployed against specific pathogens (Dewdney *et al.*, 2000).

1.9.3 Phytoalexin-deficient mutants

Pad (*p*hyto*a*lexin-*d*eficient) mutants were isolated by screening for reduced synthesis of camalexin in response to challenge by virulent *P.syringae* (Glazebrook and Ausubel, 1994). The *pad4* mutant displays reduced camalexin synthesis and *PR-1* expression in response to infection by virulent *P. syringae maculicola* ES4326 but not in response to an avirulent isolate carrying *avrRpt2* (Zhou *et al.*, 1998). SA treatment restores camalexin synthesis and *PR-1* expression. It was proposed that PAD4 is required for amplification of weak signals derived from infection by virulent pathogens and leading to the activation of SA signalling (Jirage *et al.*, 1999). The predicted PAD4 protein has similarity to the same class of eukaryotic lipases as EDS1 and they were shown to interact in two-hybrid analysis (Feys *et al.*, 2001).

1.9.4 Constitutive SAR mutants

Several mutants that display constitutive SAR have been isolated. The *cpr1*, *cpr5* and *cpr6* mutations cause abnormal plant development, elevated SA levels, constitutive expression of *PR-1*, *PR-2* and *PR-5* genes and resistance to avirulent strains of *P.syringae* and *P.parasitica* (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998). In all cases *nahG* suppresses the resistance phenotype, indicating that the CPR gene products act upstream of SA. In *cpr1*x*npr1* and

cpr5xnpr1 double mutants, *PR*-gene expression and resistance to *P.syringae* is blocked, suggesting that both CPR1 and CPR5 function upstream of NPR1. However, in *cpr6xnpr1* plants, constitutive expression of *PR-1*, *PR-2* and *PR-5* is retained but resistance to *P.syringae* is lost (Clarke *et al.*, 1998). This finding uncovered the existence of an SA-dependent but NPR1-independent pathway for activation of *PR*-genes. It also implies that resistance to *P.syringae* in *cpr6* is not caused by *PR-1*, *PR-2* and *PR-5* expression. Additionally, the *npr1* mutation does not suppress SA synthesis in *cpr* mutants. The SA concentration in *cprxnpr1* mutants was increased when compared to that found in *cpr* single mutants, suggesting that *npr1* is defective in feedback regulation of SA accumulation (Clarke *et al.*, 2000). Epistasis analysis showed that effectively, SA mediates both NPR1-dependent and NPR1-independent resistance in the *cpr* mutants and that NPR1-independent resistance requires sensitivity to JA/ET (Clarke *et al.*, 2000).

Inactivation of a MAP kinase in the *mpk4* mutant resulted in constitutive SAR, elevated SA levels and increased resistance to virulent pathogens. SAR in *mpk4* is dependent on SA accumulation but is independent of NPR1. Gene induction by jasmonate was blocked in *mpk4xnahG* suggesting a role for MPK4 in JA-dependent signalling (Petersen *et al.*, 2000).

1.9.5 Enhanced disease resistance mutants

Four mutants named *edr1* through *edr4* were isolated in a screen for enhanced resistance to virulent *P.syringae* DC3000 (Frye and Innes, 1998). The *edr* mutants differ from other types of heightened resistance mutants in that they do not display constitutive defence responses. *Edr1* was subsequently shown to be resistant to *Erysiphe cichoracearum* (powdery mildew) (Frye *et al.*, 2001). Significantly, *edr1* does not display constitutive expression of *PR-1* indicating that resistance is not caused by constitutive activation of defence genes but rather by a more rapidly induced defence response. The *edr1* gene was found to encode a putative MAP kinase kinase kinase (MAPKKK) similar to CTR1, a negative regulator of ET responses in *Arabidopsis* (Frye *et al.*, 2001). By analogy to the CTR1 model, the authors predict that two signals must be perceived before the SA pathway is turned

on, one to remove the negative regulators (EDR1 pathway) and another to activate positive regulators (for instance the EDS1/PAD4 pathways). This mechanism would explain why *edr1* plants do not constitutively express defence responses and why *eds1* and *pad4* mutations suppress *edr1* resistance (Frye *et al.*, 2001).

1.10 Other signalling components

The molecular cloning of three genes involved in the regulation of cell death has shed light on the link between HR, SA and resistance. The *LSD1* gene from *Arabidopsis* encodes a novel class of zinc finger protein that could act as a negative regulator of cell death (Dietrich *et al.*, 1997). The lethal leaf spot (*LLS1*) gene from maize encodes a putative dioxygenase that could be responsible for the detoxification of signals generated during cell death (Gray *et al.*, 1997). The *MLO* resistance gene from barley encodes a seven transmembrane domain protein that could fuction as a receptor (Buschges *et al.*, 1997; Devoto *et al.*, 1999). *MLO*-conferred resistance is histologically distiguishable from *R*-gene mediated cell death and mutants exhibit broad spectrum resistance to all known races of the powdery mildew *Erysiphe graminis* f.sp. *hordei* through de-repression of cell death and defence pathways that act at an early stage of fungal infection (Buschges *et al.*, 1997).

The *acd* mutants have been sorted into those that accumulate camalexin, an *Arabidopsis* phytoalexin and those that do not (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1997). Since camalexin production had not been previously associated to SAR, these results indicate that some lesion-mimic mutations may affect other defence-related pathways. Two recently isolated dominant mutants *acd6* and *ssi1* also show lesion formation and enhanced resistance to pathogens (Rate *et al.*, 1999; Shah *et al.*, 1999). Similar to *lsd6* and *lsd7*, they are both suppressed in a *nahG* background. Treatment with BTH not only restores the mutant phenotypes but also induces tumor-like abnormal growths in *acd6*x*nahG* plants. This observation indicates cellular growth processes can be affected by SA-dependent cell death. In an *npr1* background, both *ssi1* and *acd6* retain *PR*-gene expression and pathogen

resistance suggesting they are involved in an NPR1-independent pathway (Rate *et al.*, 1999; Shah *et al.*, 1999).

Biochemical approaches used to identify components of the SA signal-transduction pathway have shown that protein kinases and phosphatases are crucial for activation of early defence responses (Yang *et al.*, 1997; Scheel, 1998). Involvement of mitogen-activated protein kinases (MAPKs) in *R*-gene mediated signalling was first demonstrated in TMV-infected tobacco plants (Zhang and Klessig, 1998). Plants carrying the tobacco *N* gene were able to induce two MAP kinases, a 48 kD salicylic induced protein kinase (SIPK) and a wound induced protein kinase (WIPK) (Zhang and Klessig, 1998). Calmodulin antagonists were later shown to inhibit MAPK activation implicating calcium-dependent protein kinases (CDPKs) in the signalling pathway (Romeis *et al.*, 2000).

1.11 Jasmonate/Ethylene signalling pathway

Aside from SA, the plant hormones JA and ET are thought to play a key role in the deployment of defence responses (Creelman and Mullet, 1997). JA and its derivatives are synthesized from linolenic acid via an inducible octadecanoid pathway regulated by lipoxigenase (*LOX*) activity (Blechert *et al.*, 1995). JA, its methyl ester and aminoacid conjugates can be detected at low concentrations in unperturbed plant tissues (Sembdner and Parthier, 1993). They are involved in many physiological processes including root growth, tendril coiling, leaf senescence, stomatal opening, pollen formation and defence responses against insects and pathogens (reviewed in Creelman and Mullet, 1997). However, the local and systemic JA accumulation in wounded or in fungal elicitor treated plants is a tightly regulated process (Bell and Mullet, 1993). JA acts as a signalling molecule which induces the expression of genes encoding PR-proteins such as defensins (Penninckx *et al.*, 1996) and thionins (Epple *et al.*, 1995), proteinase inhibitors (Farmer *et al.*, 1992) and enzymes in phytoalexin metabolism (Creelman and Mullet, 1997).

ET is produced from methionine by the activity of ACC (1-aminocyclopropane-1-carboxylic acid) synthase and ACC oxidase (Bleecker *et al.*, 1986). ET also regulates diverse metabolic and developmental processes in plants, among them senescence of plant organs, plant growth, fruit ripening and response to abiotic and biotic stress (Abeles *et al.*, 1992). It exhibits various morphogenetic effects that have been used for the identification of *Arabidopsis* mutants with altered ET biosynthesis or sensitivity. This test is the so-called triple response and consists of agravitropic (horizontal) growth, inhibition of hypocotyl and root elongation, and thickening of the stem (Ecker, 1995). In addition to JA, ET has been shown to be required, but not sufficient to induce the wound-response (O'Donnell *et al.*, 1996) indicating that ethylene is a modulator rather than an a controller of defence signalling.

One of the best studied responses involving JA and ET is the resistance to insect herbivory (McConn *et al.*, 1997). It is triggered by wounding and insect feeding and results in the induction of proteinase inhibitors that interfere with digestion in the insect gut and discourage further feeding (Pena-Cortes *et al.*, 1995). JA/ET-signalling is required in this system, as plants that are defective or insensitive to these hormones are also super-susceptible to insect predation (Reymond and Farmer, 1998). Exogenous application of SA has been reported to inhibit the synthesis of JA and the expression of JA-regulated genes (Pena-Cortes *et al.*, 1993; Doares *et al.*, 1995). Conversely, it is possible that coronatine, a JA analogue of bacterial origin is used by the pathogen to suppress SA-mediated resistance (Feys *et al.*, 1994).

Despite the documented antagonism between microbially induced and herbivore induced pathways, evidence accumulated suggests that the JA/ET pathway plays a role in defence against specific fungal pathogens. For example, the JA-insensitive mutant *coi1* and ET-insensitive mutant *ein2*, display enhanced susceptibility to infection by the necrotrophic fungus *Botrytis cinerea* (Feys *et al.*, 1994; Thomma *et al.*, 1999). Furthermore, *coi1* and *ein2* mutants do not display enhanced susceptibility to *P.parasitica,* which is known to engage SA-dependent defence responses (Thomma *et al.*, 1999). Additionally, the JA-insensitive mutant *jar1* and the JA-deficient triple mutant *fad3-2fad7-2fad8* are more susceptible than wild-type plants

to the root pathogen *Pythium irregulare* (Staswick *et al.*, 1998; Vijayan *et al.*, 1998), indicating that both JA and ET are necessary for resistance against some necrotrophic pathogens.

JA biosynthesis is stimulated locally and systemically following pathogen infection (Penninckx *et al.*, 1996). JA and ET were shown to co-regulate a specific set of *PR* genes in *Arabidopsis* including *PDF1.2* (*p*lant *de*fensin), *Thi2.1* (*thi*onin) and *CHI-B* (basic *chi*tinase) (Epple *et al.*, 1995; Thomma *et al.*, 1998; Norman-Setterblad *et al.*, 2000). For induction of *PDF1.2*, both JA and ET are required simultaneously, as opposed to sequentially and application of SA does not induce the expression of this gene (Pennickx *et al.*, 1998). The *cev1* mutant showed constitutive expression of *VSP1* (*v*egetative *s*torage *p*rotein), *PDF1.2*, *Thi2.1* and *CHI-B* and had enhanced resistance to several species of *Erysiphe* (Ellis and Turner, 2001). The *cev1* phenotype also required both *COI1*, a component of the JA-signalling pathway and *ETR1*, which encodes an ethylene receptor (Ellis and Turner, 2001).

In contrast to the above observations, there is also evidence that the SA-dependent and JA/ET-dependent pathways might be co-regulated. For example, many lesion-mimic mutants express both SA-dependent and JA-dependent pathogenesis-related genes. Other mutants express both pathways constitutively in the absence of HR-cell death, suggesting that co-regulation is not the result of activation of the wound response by cell damage (Maleck and Dietrich, 1999). Similarly, microarray analysis indicated a significant overlap of defence-related genes that can be induced by SA and JA (Schenk *et al.*, 2000).

Another mechanism of resistance, termed induced systemic resistance (ISR), induced by some non-pathogenic rhizosphere bacteria was found to be dependent on JA/ET. Activation of this pathway does not lead to expression of *PDF1.2*, the marker for the classic JA/ET response (Pieterse *et al.*,1996). In contrast to the wound and pathogen induced JA/ET pathway, the ISR pathway requires JA and ET sequentially and intriguingly also requires NPR1 (Pieterse *et al.*,1998). A recent study showed

that ISR and SAR can be activated simultaneously, resulting in an additive level of protection against *P.syringae* (van Wees *et al.*, 2000).

1.12 ET-insensitive and JA-insensitive mutants

The ET pathway is one of the best known in plants. Several components have been characterised and placed in a linear order of interaction (Roman *et al.*, 1995). The recessive mutant *ein2* (*E*T-*in*sensitive) is able to accumulate normal levels of ET but is not responsive to endogenous or applied ET, or to the ET-precursor ACC. The cloned *EIN2* gene encodes a metal-ion transporter that belongs to the Nramp family of antimicrobial proteins (Alonso *et al.*, 1999). ET perception is also blocked in the dominant mutant *etr1* without affecting ET production (Guzman and Ecker, 1990). *ETR1* encodes a histidine kinase similar to members of the two-component receptor kinase family which function as sensors and transducers of environmental stimuli in bacteria (Chang *et al.*, 1993).

The JA-signal transduction pathway is not well known. Two mutants that block JA synthesis or perception have been identified. The *jar1* mutant was identified by a loss of JA-induced root-growth inhibition and excessive anthocyanin accumulation (Staswick *et al.*, 1992). The recessive, male-sterile mutant *coi1* (coronatine-insensitive) is insensitive to the *P.syringae* toxin, coronatine and was subsequently shown to be unable to perceive JA (Feys *et al.*, 1994). The *COI1* gene has been cloned and encodes a putative F-box protein involved in the ubiquitination pathway (Xie *et al.*, 1998). It may function by degrading a negative regulator in the JA-signalling pathway (Xie *et al.*, 1998).

1.13 Overview of contents

The broad aim of this study was to investigate the regulation of *PR-1*, a gene that plays a key role in resistance against pathogens in *Arabidopsis*. Two different approaches were undertaken. In the first project, a novel basic *PR-1* gene was isolated and characterised. As a first step towards the identification of *cis*-regulatory elements involved in the control of *AtPRB1* expression, a set of promoter deletions were analysed in transgenic plants. The LUCIFERASE reporter system was used to characterise the temporal and spatial patterns of *AtPRB1* promoter activity in different plant organs. In addition, inducibility by ET, Me-JA and SA was investigated and a number of promoter regions important for both the establishment of organ-specific expression and responsiveness to ET and Me-JA were defined. Finally, a putative function for AtPRB1 was inferred from these results.

In the second project, a genetic approach was used to isolate *Arabidopsis* mutants involved in the regulation of acidic *PR-1* expression. A mutant screen strategy was described that enabled us to identify four SAR mutants displaying constitutive *PR-1* gene expression and enhanced resistance to *P.parasitica* Noco2, a *Peronospora* isolate that is virulent on wild-type plants. Preliminary characterisation of the mutants designated *esr2*, *esr3*, *esr5* and *esr6* (for *e*nhanced *s*ystemic *r*esistance) suggested the involvement of multiple pathways in resistance mediated by *esr* mutations. Analysis of *PR-1* accumulation in the lesion mimic mutant *esr2* and *esr2* lines introgressed into different mutant backgrounds indicated that SA is necessary but not sufficient for full *PR-1* induction. The possibility that ESR2 may function as a regulator of a signalling pathway that requires SA, JA and ET was discussed. Finally, the significance of the research and potential impact in agriculture were considered.

CHAPTER 2

Materials and Methods

2.1 *Cloning of the Arabidopsis AtPRB1 gene*

An *Arabidopsis* Ws genomic DNA library (CD4-11, pOCA18 binary cosmid library) obtained from the *Arabidopsis* Stock Center, Ohio (Schulz *et al.*, 1994) was screened with a labelled *Arabidopsis PR-1* cDNA probe (Uknes *et al.*, 1992) under standard hybridisation conditions (Maniatis *et al.*, 1982). DNA from a positive clone was isolated and subjected to restriction enzyme analysis followed by hybridisation with the same *PR-1* probe. A 5 kb *EcoRV/SalI* fragment was subcloned into pBluescript SK (Stratagene) and further digested with *PstI* (contribution by Thomson, C.). The resulting 3.5 kb and 1.5 kb fragments were separately cloned into Bluescript SK for further manipulation and sequencing.

2.2 *AtPRB1::LUC constructs and sequencing*

The *Photinus pyralis LUC* gene was released from plasmid pSP-*LUC*+ (Promega) with *Hind* III and *Xba* I. To generate pART7*LUC*, the CaMV 35S promoter was first excised from pART7 (Gleave, 1992) using *Sac* I and *Xba* I restriction sites. *LUC* was then cloned into pART7 and an *Nco* I restriction site was introduced to facilitate an N-terminal fusion with the promoter. The *AtPRB1* promoter fragment was amplified by PCR using the following set of primers: 5′-CTAGAGCTCGTTGGGTTTATTAT AGTAC-3′ and 5′-GGTACCAAGAATCAACTTTATTACCGATA-3′. The amplified product was subsequently digested with *Sac* I and *Nco* I and cloned into pART7*LUC* digested with the same restriction enzymes. Thus, the resulting plasmid pART7*AtPRB1*::*LUC* contained the *AtPRB1* promoter fragment fused to the *LUC* gene and adjacent OCS termination sequence (MacDonald *et al.*, 1991) within

pART7.A set of nested 5'-deletions was then produced to complete the sequencing of the *AtPRB1* promoter fragment. Deletions were generated with exonuclease III as described by Henikoff (1984). A pBluescriptSK *Bam* HI restriction site at the 5′-end of the *AtPRB1* promoter fragment was used to generate 5′-overhang substrates for exonuclease III, while *Bst* XI digestion beyond the 3′-end of the OCS sequence produced 3′-overhangs which are not digested by exonuclease III. A series of overlapping 5′- deletions were subsequently identified using standard procedures (Henikoff, 1984). The chosen deletion fragments were religated in pART7 and the rubidium chloride method was used for transformation of competent *E.coli* (Sambrook et al., 1989).

Sequencing of this series of *AtPRB1* promoter deletion mutants was performed using a Hybaid Omnigene Thermocycler using the Perkin-Elmer ABI prism dye terminator cycle sequencing ready reaction kit, according to the manufacturers instructions. Dry products were run on an ABI 377 automated DNA sequencer (ICMB sequencing services). Results were analysed using Perkin-Elmer ABI Prism Editview or Genejockey packages for the MacIntosh. The sequence data was transferred to the UNIX-based GCG program for sequence alignments. Amino acid sequence alignments were undertaken using the ClustalW programme.

2.3 *Northern analysis of AtPRB1*

For Northern blot analysis RNA was isolated from 4-week old plants using the RNAeasy Plant Mini Kit (Qiagen). Thirty micrograms of total RNA samples were separated by electrophoresis through a 1% formaldehyde-agarose gel, transferred onto a nylon membrane and immobilised by UV cross-linking (Amersham). A gene specific probe was generated for *AtPRB1* by amplifying a DNA sequence in the 3′-untranslated region using the following primers: 5′-CCTGCACAAAGGACGTGAA TAT-3′ and 5′-CTCTCAACAGCCACTAAGGTG-3′. The probe was labelled labelled with α-^{32}P-dCTP by random priming using a Prime-a-Gene labelling kit

(Promega). Hybridisation was performed according to the manufacturer's instructions (Amersham). After overnight incubation, the membrane was washed for 20 min at room temperature in 2 X SSC, 1% (w/v) SDS, followed by two 20 min washes at 65°C in 1 X SSC, 0.5% (w/v) SDS.

2.4 *Generation of transgenic plants*

The *AtPRB1* promoter and selected deletion constructs (2345 bp, 989 bp and 319 bp) fused to the *LUC* reporter gene were released from plasmid pART7*AtPRB1*::*LUC* by *Not* I digestion and cloned into the *Not* I site of binary vector pART27 (Gleave, 1992). The resulting recombinant plasmids were used to transform *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) by the freeze and thaw method (Chen *et al.*, 1994), which was subsequently employed to generate transgenic *Arabidopsis* plants of the Col-0 ecotype via the floral dip transformation method (Clough and Bent, 1998).

2.5 *Chemical treatments*

To assay gene expression in aerial tissues, *Arabidopsis* T_2 plants were grown in soil at 22°C using a 10 hour photoperiod. Alternatively, *Arabidopsis* plants were grown from sterilised seeds in MS liquid culture to assay root gene expression. A 10 mM salicylic acid solution in phosphate buffer (pH 5.8) was added to root MS liquid cultures to give a final concentration of 0.1mM. For Me-JA treatment, a 10mM Me-JA solution containing 0.05% ethanol was added to the liquid culture media to a final concentration of 50μM. To assay for possible ethylene induction, 5mM ethephon in phosphate buffer was exogenously added to *Arabidopsis* plants grown in liquid culture. Following all treatments, leaves and roots were assayed after 24 hours.

2.6 *Expression of the AtPRB1 protein*

A PCR fragment containing the coding region (+1 to +484) of the *Arabidopsis ATPRB-1* gene was cloned into the *Bam* HI/*Nco* I sites of the bacterial expression vector pRSETB (Invitrogen). Primer sequences used for PCR were 5'-AATTTCCGGAGATTTTGG-3' and 5'-CATCTTGGTTTTATACAC-3'. This construct was initially transformed into *E. coli* DH5œ, then transferred into *E. coli* BL21 (DE3) PLysS (Promega) for ATPRB-1 protein expression. Transformation of BL21 colonies was confirmed by PCR using pRSETB primers 5'-TAATACGACTCACTATAGGG-3' and 5'-TGCTAGTTATTGCTCAGCGGT-3'. Expression of recombinant protein was carried out according to the Invitrogen instructions. Cultures of transformed colonies were grown at 37 °C to stationary phase (OD_{600} = 0.4-0.6), then induced with 1 mM IPTG and incubated for another 5 hours. Cells were harvested by centrifugation, resuspended in the recommended lysis buffer and sonicated (3x15sec) for SDS-PAGE analysis. Electrophoresis was performed on polyacrylamide gels using a modified SDS-PAGE Laemmli system for separation of low molecular weight proteins (Ausubel *et al.*,Electrophoresis in Tris-Tricine Buffer Systems, In: Current Protocols in Molecular Biology).

2.7 *Activation-tagging mutagenesis and subsequent screen*

Agrobacterium GV3101 transformed with the pSKI015 vector (gift from I. Kardailsky) was grown overnight at 30°C in LB media containing 50 mg/l kanamycin (to select for the helper plasmid pMP90) and 50 mg/l ampicillin (to select for the pSKI015 binary vector) and used to transform *PR-1*::*LUC* by floral dip method (Clough & Bent, 1998). Seed harvested from transformed plants was sown in flats and selected by 100mg/l Basta (Agrevo) spraying two weeks after germination, then two times at four-day intervals. Resistant plants were visually inspected for any abnormalities, sub-planted and T_1 seed collected in individual lines. The mutant screen was carried out by sowing pools containing approximately 20 seeds from 60 different T_2 lines in plug-hole trays. Two weeks post-germination

seedlings were painted with luciferin, the substrate for the *LUC* gene and imaged under the camera. A set of 5000 lines was imaged for constitutive LUC activity and another identical set was imaged for no LUC activity following BTH (1mM) induction.

2.8 *Isolation of plant DNA*

Four-week old plants were used for DNA extractions. Leaf tissue was ground in liquid nitrogen and extraction buffer (100mM Tris-HCl (pH8), 1.4M NaCl, 20mM EDTA (pH8), 2% CTAB, 0.2% β-mercaptoethanol) was added to a ratio of 1g tissue: 2 mls extraction buffer. The tubes were then incubated at 65°C for 10 minutes and left to cool at room temperature for another 10 minutes. An equal volume of chloroform was added to each tube, mixed, and tubes were centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and chloroform extractions were repeated if necessary. The DNA was precipitated by adding an equal volume of isopropanol and spinning for 10 minutes at 6000 rpm. The DNA pellet was washed with 70% ethanol, allowed to dry partially and then resuspended in TE buffer or sterile water containing RNAse A to a concentration of 20 µg/ml. DNA concentration was estimated from an ethidium bromide-agarose gel using a molecular weight standard.

2.9 *Southern analysis of mutant lines*

DNA (10µg) isolated from each mutant line was digested overnight using the restriction enzyme *EcoRI* (Promega). Digested samples were run through a 0.8% agarose gel and transferred onto a nylon membrane (Amersham) following standard procedures. The membrane was prehybridised for 1 hour at 42°C and hybridised sequentially with two probes: a 339 bp fragment corresponding to the CaMV 35S enhancer region and a pBluescriptSK region. Probes were obtained by PCR using primers 5'-GATCCCCAACATGGTGGAGCACG-3' and 5'- TAGATATCACATC

AATCCACTTGC-3'(CaMV 35S enhancer); 5'-GATCCACTAGTTCTAGAGCG-3' and 5'-GTCATTCTGAGAATAGTGTAT-3'(pBluescriptSK). Hybridisation and chemiluminescent detection using a DIG-DNA labelling kit (Boehringer) were performed as described by Spangenberg (Potrykus *et al.*,1993).

2.10 *Fungal pathogenicity assays*

Peronospora parasitica Noco2 (gift from Jane Parker) was maintained on Col-0 seedlings grown in magenta jars. For the *P.parasitica* disease resistance assays, conidiospores were harvested by vortexing infected seedlings in water. Spore concentration was determined using a haemocytometer, and resuspended in sterile distilled water to $1x10^5$ spores per ml. Four-week old plants grown under short day conditions were sprayed with the conidiospore solution and placed in trays covered with clear lids to maintain a humid environment. Fungal growth on plant leaves (visualised as condiophore growth) was scored 10 days post-infection using a qualitative method adapted from Cao et al. (1997). Scoring was as follows: 0 - no infection, 1 - less than 25% of one leaf with conidiophore growth, 2 - 25 to 50% of one or two leaves covered with condiophores, 3 - 25 to 50 % of three or four leaves covered with conidiophore growth, 4 - 25 to 50% of all leaves covered with condiophore growth, 5 - all leaves covered with conidiophore growth. Plants in different replicates were assigned a disease index as follows: $D.I.=\sum iXj/n$, where i=infection class, j=the number of plants scored for that infection class and n=the total number of plants in the replicate (based on Epple *et al.*,1997). For the spore count assay, conidiospore suspensions of *P.parasitica* Noco2 (10^5/ml) were sprayed onto 10-day old seedlings grown in magenta boxes. Leaves from 20 plants in 5 independent samplings were collected 7 days after inoculation. A spore suspension was obtained by vortexing leaves in 2 mls of dH_2O and the number of spores was counted using a haemocytometer 5x5 grid in a Leica Wild M3C microscope.

2.11 *Bacterial pathogenicity assay*

P.syringae pv *tomato* (*Pst*) DC3000 was grown in King's broth (KB) liquid media supplemented with 50 mg/l rifampicin. Four week old soil-grown plants were infected with a *Pst* DC3000 suspension (OD_{600}= 0.0002) in 10 mM $MgCl_2$ by completely infiltrating the abaxial surface of the leaf with a 2 ml syringe (Cao *et al.*, 1994). Three leaves per plant, and three plants per line were infiltrated. After three days, plants were inspected for development of symptoms. Leaves were also harvested at this point for analysis of bacterial growth. Leaf discs of uniform size (0.5 cm^2) were taken from the leaves using a cork borer. Three leaf disks from each plant were ground in 990µl 10 mM $MgCl_2$ in a pestle and mortar. Serial dilutions were made from the resulting bacterial suspension and 100µl of each dilution was inoculated onto KB plates. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies per sample was recorded. Col-0 plants sprayed with BTH (300µM) were used as control for induced resistance.

2.12 *Luciferase imaging and assays*

Leaves of *AtPRB1*::*LUC* or *PR-1*::*LUC* transgenic plants were painted with a solution containing 1mM Luciferin (Biosynth AG), and 0.01% triton X-100 and 0.03% Silwet (Union Carbide) in a 1mM sodium citrate buffer (pH 5.8). All *in planta* LUC imaging was performed using an ultra low light imaging camera system (EG & G Berthold Luminograph 980). Images were routinely collected over a 30s (*AtPRB1*::*LUC*) or 10s (*PR-1*::*LUC*) time period. LUC assays *in vitro* were performed according to the manufacturer's instructions (Promega E1500) using a luminometer (EG & G Berthold LB96P microplate luminometer) to measure light emission. LUC activity was determined over a 0.5s time period. The protein concentration of each sample was determined by Bradford Micro-Assay (BioRad).

2.13 *Cross-pollination and selection of mutant backgrounds*

Genetic crosses were undertaken by dissecting and emasculating unopened flower buds and then using the remaining pistils as recipients for pollen from opened flowers. Transgenics with a selectable marker (i.e. kanamycin or BASTA) were always used as the pollen donor and mutant lines as recipient. Successful crosses were allowed to self and homozygous transgenic plants were screened from their progeny. Progeny containing *nahG* were identified by the appearance of brown deposits in root tissue when grown on MS media containing 0.5mM salicylic acid (Bowling *et al.*, 1994). The ethylene insensitive mutant *etr1* was selected at 2-3 weeks on MS plates containing 10μM ACC by virtue of having significantly longer roots than wild-type plants. Mutants successfully introgressed into the *coi1* background were selected by normal growth on MS plates containing 100μM Me-JA in comparison to stunted wildtype plants. Appropriate controls were always used to check selection method.

2.14 *Northern analysis of esr mutants*

Samples (10 μg) were separated on formaldehyde-agarose gels (Sambrook *et al.*, 1989), transferred to a Hybond™-N hybidization membrane according to the instructions of the supplier (Amersham Lifesciences) and hybridised with a *PR-1* probe (Uknes *et al.*, 1992). The *PR-1* gene sequence was initially isolated by PCR from an *Arabidopsis* cDNA library and cloned into the TA vector (Thomson & Loake, unpublished). The 0.3 kb fragment used as *PR-1* probe was obtained by PCR using primers 5'-CTGCAGACTCATACACTCTGG-3' and 5'-TATGTACGT GTGTATGCATGATC-3'. In order to identify lane-to-lane variations in the amount of RNA added and thus facilitate comparisons between lanes, blots were probed with the ribosomal 18S (r18) probe. It was obtained by PCR from pBluescript-18S using T7 and T3 primers (Pruitt and Meyerowitz, 1986). Probes were labelled with α-^{32}P-

dCTP by random priming using the Prime-a-Gene® labelling system (Promega). Dextran sulphate (10% w/v) was included in the pre-hybridisation / hybridisation solution for efficient binding of the probe (Sambrook *et al.*, 1989). Blots were washed twice for 30 min each at 65°C in 4 X SSC, 1% (w/v) SDS, followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate time period and stripped by incubation in boiling 0.1% (w/v) SDS and washed in 0.5 X SSC for 30 min at room temperature, before hybridisation with a subsequent probe (Sambrook *et al.*, 1989). Methylene blue staining was used to monitor loading in the northern blot of *esr2* double mutants. The blot was incubated for 5 min in a 0.3M sodium acetate (pH 7) and 0.03% methylene blue solution and washed several times with dH_2O until bands were clearly seen. After the image was scanned, the blot was washed for 30 min with warm 1 X SSPE, 0.1% (w/v) SDS and prehybridised.

2.15 *Ethylene determination*

The ET efflux was determined by noting the increase in concentration over a collection period of 3 hours. Measurement of the accumulated ET at 30 min intervals over 5 hours confirmed that accumulation remained linear. Plants grown on soil for three weeks were removed from pots, soil was washed off and roots were individually wrapped in wet paper. Five samples were collected from weighed plants using 20 ml Plastipak syringes. The syringes containing the plants were sealed and incubated in the light for 3 hours. Gases were analysed by gas chromatography using an HP 5890 series II gas chromatograph with a flame ionisation detector. Porapak QS columns were used at 50°C.

2.16 *Trypan blue staining*

Leaf samples were boiled for 2 min in lactic acid-phenol-trypan blue solution (2.5% mg/ml trypan blue (w/v), 25% lactic acid (v/v), 23% phenol (v/v), 25% glycerol and water (v/v) (Bowling *et al.*, 1997). After allowing the solution to cool down at room temperature, the trypan blue staining solution was replaced with a chloral hydrate solution (25 g in 10 ml water) for overnight de-staining. The chloral hydrate solution was poured off and the samples were equilibrated in 70% glycerol (v/v) in water and mounted onto microscopic slides. Stained leaves were viewed for micro-lesions or fungal hyphae and spores using a Leica Wild M3C microscope.

CHAPTER 3

Isolation and characterisation of *AtPRB1*, a novel basic *PR1* gene in *Arabidopsis*

3.1 Introduction

PR-1 is the predominant type of PR-protein induced by pathogens or SA in tobacco and *Arabidopsis*, and is commonly used as a marker for the establishment of SAR (Malamy *et al.*, 1990; Uknes *et al.*, 1993; Dempsey *et al.*, 1997). The PR-1 family comprises several acidic and basic isoforms. Biochemical and immunolocalisation studies showed that three acidic isoforms from tobacco (PR-1a, -1b and –1c) are synthesised both locally and systemically by specialised cells known as crystal idioblasts and accumulate in the extracellular spaces surrounding vascular bundles (Dixon *et al.*, 1991). On the basis of electrophoretic activity and sequence homology, additional basic PR-1 counterparts have been identified. The p14 protein of tomato, which is related to the basic PR-1g of tobacco was localised within the vacuoles of leaf cells and also in the extracellular space of viroid-infected tomato plants (Vera *et al.*, 1989). The differential accumulation of p14 in vacuoles and extracellular space is thought to result from vacuole leakage following pathogen-induced cellular disruption. Generally, basic PR isoforms accumulate at relatively low levels in the vacuole and are not only induced upon infection but are also expressed in a tissue-specific and developmentally-controlled manner (Eyal *et al.*, 1993; Linthorst, 1991). In contrast to acidic PRs, many basic PRs are not deployed in response to SA and are not expressed in systemic tissues. Therefore, the accumulation of these proteins does not correlate with the establishment of SAR (Niki *et al.*, 1998). In tobacco, basic PR1 proteins are thought to be encoded by a gene family of up to 8 members (Cornelissen *et al.*, 1987). Characteristically, their expression can be activated following the accumulation of ethylene, which is superimposed upon organ-specific expression in flowers, stems and roots (Memelink *et al.*, 1990). Moreover, induction of basic *PR-1* gene expression and other basic *PR* genes in tobacco is triggered by Me-JA (Niki *et al.*, 1998).

PR-1 was found to possess anti-fungal activity against oomycetes. Constitutive expression of PR1-a in tobacco caused enhanced protection against *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al.*, 1993) but not against the non-oomycete *Cercospora nicotianae* or the bacterial pathogen *Pseudomonas syringae* (Alexander *et al.*, 1993). Additionally, different PR-1 family members from tomato and tobacco displayed inhibitory activity on the growth of *Phytophthora infestans* in tomato leaf disc assays, with the tomato basic PR-1c and tobacco basic PR-1g being the most effective isoforms (Niderman *et al.*, 1995). Their limited antifungal activity implicates the PR-1 family in plant defence but their biochemical activity or relationship to other proteins is still unknown.

A nuclear magnetic resonance structure of tomato PR-1b revealed a unique molecular architecture (Fernandez *et al.*, 1997). Four α-helices are located on both sides of a central β-sheet anti-parallel arrangement and are stabilised by hydrogen bonds, resulting in a compact molecular core. This compact α-β-α structure is shared by all the PR-1 family members and reflects their high stability and their insensitivity to several proteases (Van Loon and Gerritsen, 1989). Related sequences were found in proteins from yeasts, insects, nematodes and vertebrates. These include allergens of ant and vespid venoms, snake and lizard toxins, vertebrate cysteine-rich secretory proteins (CRISPs) and a human brain tumor protein (GliPR) (Lu *et al.*, 1993; Morrissette *et al.*, 1995; Hayashi *et al.*, 1996; Szyperski *et al.*, 1998). The insect allergens as well as the reptile toxins are likely to have evolved for use against other insects or vertebrates (Hoffman, 1993). It has been speculated that CRISPs might encode lytic enzymatic activity. The mouse CRISP-3 protein is expressed in the male salivary gland (Haendler *et al.*, 1993) and in developing immune system cells, where a possible lytic activity might be related to an antimicrobial function in saliva and in the blood or lymph (Pfisterer *et al.*, 1996). The glioma pathogenesis-related protein (GliPR) is expressed in the predominant human brain tumor and can be induced in macrophages which are phagocytic immune system cells, but is not detectable in normal tissues (Szyperski *et al.*, 1998). Its high expression in tumor cells has been proposed to be related to their malignant properties (Szyperski *et al.*, 1998). The widespread occurrence and possibly common function in defence suggests that in

addition to structural similarities, plant, insect and vertebrate PR-1 proteins share functional similarities (Van Loon and Van Strien, 1999). Furthermore, the striking conservation of the putative active site residues among evolutionarily divergent species, has been proposed to indicate that the entire PR-1 "superfamily" originated from a common ancestor and operates according to the same molecular mechanism (Szyperski *et al.*, 1998).

Despite their apparent importance in the establishment of disease resistance, only two *PR1* genes, encoding acidic isoforms, have been described to date in *Arabidopsis* (Metzler *et al.*, 1991; Uknes *et al.*, 1992). We have isolated and characterised a gene encoding a basic PR1-like protein from *Arabidopsis*, designated *AtPRB1*. In order to gain insight into the transcriptional regulation of this gene, we investigated the temporal and spatial expression using a promoter-reporter gene (*LUC*) fusion in transgenic *Arabidopsis* plants. To further study the structure of the *AtPRB1* promoter, a set of deleted promoter-reporter constructs were generated that delineated the regions containing *cis*-elements involved in the regulation of gene expression. Finally, we assessed the inducibility of *AtPRB1* to SA, Me-JA and ET, key cues in the development of disease resistance.

3.2 Cloning and characterisation of the *Arabidopsis AtPRB1* gene

A cDNA encoding an *Arabidopsis* PR1 protein (Uknes, 1992) was used to screen an *Arabidopsis* genomic library (Schulz *et al.*, 1994). One of the cross-hybridising colonies was subsequently purified to homogeneity. Plasmid DNA from this colony was isolated, digested with a series of restriction enzymes, Southern blotted and hybridised against a labelled *PR1* probe. Two cross-hybridising *Pst* I fragments of approximately 3.2 and 1.5 kb were subsequently cloned into pBluescript. A series of 5′-deletions were generated within the largest *Pst* I fragment using exonuclease III to augment rapid sequence determination (Genbank accession number AC005398).

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TGGAGTAACAAATAGCCAAACACGCAACCCAAATAATATCATGGCTCATCATTACGATTC   -2310
TTTTTCTGTAACTTACGCCGGCGGGATTTTTTGGCAGGTTTTTGTCGCCGCATTAAATGC   -2250
GCAATATCCATATTTCCCAAATCTTTTTTCAGTCCCCTACAAGTATCCTGAACAATAGGG   -2190
TTAAAATAGGAATATTCGTTTTGCATGATTTATCACACCTAAATATCTGATTTACATATA   -2130
TATATTCTTAATTAACTCACGCTGCATGTACATATATTTAACTCACCCGACCTAACGTAT   -2070
TTTATTGTTTTACATTTATTTAATTACGTATAAATTATATGAAAATAGTTTAATAATGAC   -2010
AAATTGACAGCATCCAGTATGAATAAAAAGCTATTAGACTTCTTATCTATTAGCTTAGCC   -1950
ACCATTCAATTTGTGTTTTCTTTCAAAATGATATGTCATATTTGCCTAACTTAACAAACC   -1890
TCATTCAAGAAGCAGCTTTGATTATTCCATCCCCTGCAAGAAGCAAGTAACTATTACCGT   -1830
GGAATTATTGTATCAATGACTTATTTAACAAGTGAAGATGGCTTTGTTTTATTCCCTCAT   -1770
GGATTATGCTTTATTTTTTGGAAAAAAAATACTCAGCTACACCAAGTAGAAGGGAATACA   -1710
TGGGTCACACATGCTAAGTTTAGAAATGTACAACCAACTACACGAACTAAATTTATTGCA   -1650
TACCCACATATATCAACTATATGACGATATGTGGTCAACATCAACATCACGACATGTGGT   -1590
CAATGCCGATCAACGATGGTCAACACCGGATTTCGTGATGTTGACCACATGTCGTCATAT   -1530
AGTTGGTATATGTGGGCATACAATACATCTAATTTGTGTAATTAGTTGTACATTTTTAAA   -1470
CTTGGTATGTGTGATCATGTATTTCGTCATACTTGGTGTAGCTGACTATCTTTTTTCCTT   -1410
ATTTTTTCCTTCCTTTTTTTTTATAAATCATAAAGAAAGACTTCTTGTTTTATTCCCTCA   -1350
TGGCTCCATGTTACAAACATAAGCAAGACCGTAGGAAAGAAATACATGCATGAAACACTA   -1290
TTTTCATAACATTTCACTTGTAAATATTTTCTTTTAAATAATTGCATGTATCTTTTCTCA   -1230
TAATTATTTGATTTTCTAATGGGGTATTAAATATGTCCACGGACAATATTAATTCATATT   -1170
TTAGCAGTGAGGCAAAAAGTTTTGTAATACAATGGAGACTGGAAAATGAATAAAGAAAAT   -1110
AAGCTTCCACTTTTCTCAAAAATGCTTTAAAAATCGAATTAATTATGGATTCGCCATTTT   -1050
TTTTATTGACATTTGTGGATTTTTTCTTCTTTTAAATAGTTTGATTTTGCCTTTTTTCAT    -990
AATATAAAAATGTAATTTAACGTACATTTTACTCATTATGTTTGAGTTTATTCTCTTTCA    -930
TTTGTTTTTAGAGTTTAGTATTTGATATAAGTACGTATTTTTCCAAAATAAAATCATAAA    -870
TACATATATTTTTAAATGTTAAAAATGGCAAATTCGTCAGATTTTCGTAAAAATAGCATT    -810
TTGAAAATTTTATTTTTTAATAGATTTGAAAAATTCTTACTTTTGCAGGAAAACAAAAAA    -750
TAAACTACAACATTAAAGAATGTTGAAGATATGATCGATTTTGTTAAATCAGAGATAAAA    -690
ACCACAAGTAAGAATTTTTAGGGTGCTTATGATTTGTTTTAACTGTTTTCATTATAATAT    -630
TAATCTCCAACACCACATAGATAATCACTTTATAATACGATATATGCCCATATCTAAAAT    -570
TTAGGTTGTAATAAATATTTTGGCAATTTTAGTTCAGACTATTTACAAGATTGAAGAGAT    -510
AATTATGGTTAGGCATTGGGGCTTATAAGTCATGTTCACAAAATTTATGAACTTCAGTTA    -450
TTCAATTTGAAAGAAGGGTATCCAGAGTTAGACTGAACAAAATCTTCGCTTAACTAAATT    -390
CAAAATTATAAAAATTTGGTTTGGTTGTAGTTAGATATCCCTTGCCGAATTGAACCAAAA    -330
ATTTCGATAATCTTGGTTTAAATTTGTTTGTTATTTTGTTTAGGCTGTTATATATTTTTG    -270
GTACTTTCTTATGTTTCTTTTTAAAAATTGTTTTCATATGAAGATGTGAGAAGAAATTTA    -210
GACCTATGGGTTAGGGGGTTCCCGACTAGCTACCGTATTAAATGTTGGGTTTATTTTATC    -150
TCCTATCTCCTATCCAAAGGTGAAAGATTAAACCCATAAAAGAGGTTTAAATGTTCAGCA     -90
AGACTTTGTTTATTTATTATGGCAAACAAAACGAAAGATAGTTTTCAAATCTCTATAATAA     -30
ACAATGTTTACAAACCCCAAAATCATAACACAAACAAAGCTTGGAAATAGCCATTATTTC     +31
AACTAAGAAAAATGAAAGTCACAAGCTACTCTCGAATTCTAATAATCTTGGCAGCACTTG     +91
          M  K  V  T  S  Y  S  R  I  L  I  I  L  A  A  L

TGGGTGCTCTTGTTGTTCCCTTGAAGGCTCAAGACAGCCAGCAAGACTATGTAAATGCTC    +151
V  G  A  L  V  V  P  L  K  A  Q  D  S  Q  Q  D  Y  V  N  A

ACAACCAGGCACGATCGCAGATAGGCGTAGGCCCCATGCAGTGGGACGAAGGACTTGCAG    +111
H  N  Q  A  R  S  Q  I  G  V  G  P  M  Q  W  D  E  G  L  A

CCTACGCTCGGAACTACGCAAACCAACTAAAAGGAGACTGCAGACTCGTACATTCCCGTG    +171
A  Y  A  R  N  Y  A  N  Q  L  K  G  D  C  R  L  V  H  S  R

GGCCTTACGGGGAGAACTTGGCAAAGAGTGGCGGTGACTTGTCTGGTGTTGCTGCCGTGA    +231
G  P  Y  G  E  N  L  A  K  S  G  G  D  L  S  G  V  A  A  V

ACTTGTGGGTTAACGAGAAGGCTAACTACAACTACGATACAAACACGTGCAACGGAGTTT    +291
N  L  W  V  N  E  K  A  N  Y  N  Y  D  T  N  T  C  N  G  V

GCGGTCACTACACTCAGGTCGTTTGGAGAAATTCTGTGAGACTCGGATGTGCTAAAGTGA    +351
C  G  H  Y  T  Q  V  V  W  R  N  S  V  R  L  G  C  A  K  V

GATGTAACAATGGTGGAACCATCATCAGTTGCAATTATGATCCTCCGGGGAACTATGCGA    +411
R  C  N  N  G  G  T  I  I  S  C  N  Y  D  P  P  G  N  Y  A

ACCAGAAGCCTTACTGATGTGTGATCATGTTACACATATGGCATATACACACACATATAA    +471
N  Q  K  P  Y

AACACGGACCTGCACAAAGGACGTGAATATATATTTCAATAAGAAGCATCATATGCAGGA    +531
TGTGTATAAATATTTATTAAAAAATACAAATAAGTTAAAGGCCTCTAAAACCAAAAGAAA    +591
ACTCTAGGCTTGCTCTAAGAAAAAAATATATTGGCATTTCTTAGAGTTAGAGCACGTAAT    +651
ATTCTTAAAAATCCGTTTCCACATATTTTGGTTCTACATTCCCGATTATTAGAATTCACC    +711
TTAGTGGCTGTTGAGAGGACAGACTCTCCCCAGATGTAGTGGCTGCGGTCGTGAATTGGG    +771
TTACCAAAGCTTGTTGTTATTCTGTTTGTGAATCATGAAAACGGTCAAAGATGATTCTAA    +831
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Figure 3.1. Nucleotide and predicted aminoacid sequence of the *AtPRB1* gene and gene-promoter. The translation start codon is highlighted in bold letters (+1). The transcription start codon is underlined. A putative TATA box sequence is present at –34.

An open reading frame was identified that encoded a protein of 161 amino acids with a predicted molecular weight of 17,542 kD (Fig.3.1). Analysis of the predicted amino acid sequence using BlastX (Altschul *et al.*, 1990) revealed this gene encoded a PR1-like protein, with greatest similarity, 78% identity at the amino acid level, to a PR1 protein from *Brassica napus* (accession U64806, Zhang and Fristensky, unpublished). This PR1 gene product exhibited significantly less amino acid identity at 64% to a basic *Nicotiana tabacum* protein, designated PRB1 (Eyal *et al.*, 1992), and to a number of acidic *N. tabacum* proteins PR1a, PR1b and PR1c at 60%, 64% and 66% identity respectively (Payne *et al.*, 1988; Ohshima *et al.*, 1990). Moreover, this *PR1* gene product also showed 77% identity with a previously isolated acidic PR1 from *Arabidopsis* (Uknes *et al.*, 1992) and 61% identity with an acidic PR1 from *Solanum tuberosum* (van't Klooster *et al.*, 1999). An alignment of these proteins using the EBI ClustalW programme is presented in Fig. 3.2. Subsequent analysis of the *Arabidopsis* genome (Arabidopsis Genome Initiative database) revealed that this *PR-1* gene is located on chromosome 2 and is a member of a family comprising 11 genes.

The predicted isoelectric point for this protein was found to be 8.9, thus this PR1-like protein is basic in nature. We have therefore designated the corresponding *Arabidopsis* gene *AtPRB1*. Analysis of the amino acid sequence for key signatures identified a prominent hydrophobic signal sequence which is commonly cleaved off upon entry in the endoplasmic reticulum (Van Loon and Van Strien, 1999). The presence of six conserved cysteine residues that form disulphide bridges and a short C-terminal extension found in basic isoforms are also characteristic features of the PR1 family (Van Loon and Van Strien, 1999). Moreover, three myristoylation sites (Grand, 1989) and a phosphorylation site (Woodgate *et al.*, 1986) were also identified in the predicted amino acid sequence of this protein. A conserved transmembrane motif suggests that AtPRB1 is a membrane anchored protein.

Figure 3.2. Predicted amino acid sequence of the AtPRB1 protein and comparisons with similar PR1 proteins from *Arabidopsis* and other plant species. Protein sequences were compared using the EBI ClustalW programme and grouped according to species. Amino acids highlighted in black are identical to those found in the AtPRB1 sequence, those highlighted in grey indicate similar substitutions. The hydrophobic signal sequence is depicted as a bold line, the three myristoylation sites as a bold double line and the single phosphorylation site as a dashed line. Conserved cysteine residues are denoted by an asterisk. Abbreviations for given protein sequences in the figure are as follows: *Brassica napus* (Bn) PR1, *Arabidopsis* (At) PR1, *Nicotiana tabacum* (Nt) Pr1a, PR1b and Pr1c and *Solanum tuberosum* (St) PR1.

3.3 Characterisation of the *AtPRB1* promoter

Analysis of the *AtPRB1* promoter sequence identified a transcription start site motif (CAC), which matches the consensus for plant transcription start sites, where the adenine within this motif is the preferred site of initiation (Joshi, 1987). A TATA motif was located at -34 to -31 which is also consistent with the distance of the TATA box from the transcription start site in most plant genes (Joshi, 1987). This composition exactly matched that of another *Arabidopsis PR1* gene (Lebel *et al.*, 1998). The sequenced genomic DNA fragment therefore contained 2345 bp 5′ of the putative transcription start and 2387 bp upstream of the predicted translational start of the *AtPRB1* gene product. Sequence analysis also identified two adjacent polyA signal motifs downstream of the coding sequence (Joshi, 1987).

To determine the developmental and environmental expression profile established by the endogenous *AtPRB1* promoter, we carried out Northern blot analysis employing a *AtPRB1* gene specific probe. Endogenous *AtPRB1* transcripts accumulated in root, stem and floral tissue but not in mature leaves (Fig 3.3). Furthermore, *AtPRB1* gene expression was not found to be induced in leaf tissue in response to an avirulent isolate of the biotrophic pathogen *Peronospora parasitica* (Holub *et al.*, 1994).

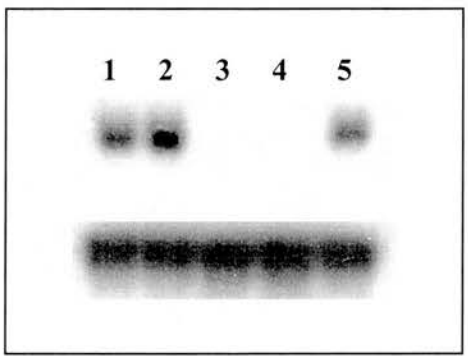


Figure 3.3. Northern analysis of *AtPRB1* expression in different plant organs and in response to attempted infection by an avirulent isolate of *P.parasitica*. RNA was isolated from flowers, lane1; stems, lane 2; leaves lane 3; *P.parasitica* challenged leaves, lane 4; and, roots, lane 5. The blot was subsequently stripped and re-probed with *Arabidopsis R18* as a control for RNA loading and transfer.

3.4 Organ-specific expression of a chimeric *AtPRB1::LUC* gene fusion

The *AtPRB1* gene promoter containing 2345 bp upstream of the putative transcription start site was fused to the coding sequence of the *Photinus pyralis LUC* gene (Fig 3.4). This fragment, referred to as full-length promoter was used to investigate promoter activity. A *Not* I fragment containing this chimeric gene was cloned into the *Not* I site of the binary vector pART27 (Gleave *et al.*, 1992) and the resulting plasmid was transformed into *Agrobacterium tumefaciens*. Subsequently, 60 independent transgenic *Arabidopsis* plants were generated that contained this construct.

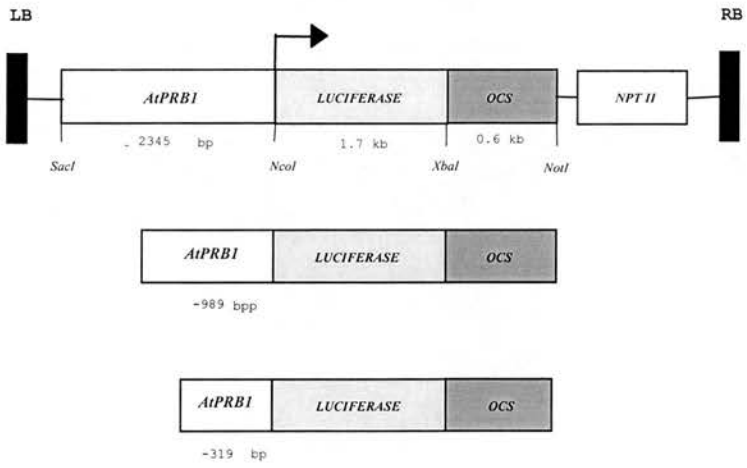


Figure 3.4. Schematic representation of the *AtPRB1::LUC* gene fusion containing -2345 bp of the *AtPRB1* promoter and the -989 and -319 bp deletion derivatives. The abbreviations utilised in the figure are: octopine synthase gene termination sequence (*OCS*), neomycin phosphotransferase gene (*NPTII*) and the right and left T-DNA border sequences (RB) and (LB) respectively. The arrow denotes the direction of transcription of the *P.pyralis* luciferase gene.

Expression of the *LUC* reporter gene was detected in 58 of the T_2 transformants examined using an ultra low light imaging camera system. Analysis of LUC activity indicated that the *AtPRB1* promoter established a well defined spatial pattern of activity in distinct plant organs. In this context, LUC activity was detected in roots, flowers and stems (Fig 3.5A, B and C). This pattern of expression reiterated that previously observed following northern analysis. Hence, the *AtPRB1*::*LUC* transgene appeared to faithfully report the activity of the endogenous *AtPRB1* promoter. To study promoter activity at different developmental stages, *in vitro*-germinated T_2 transformants were examined for LUC expression. High levels of LUC activity were found exclusively in meristems of 1 week-old plants (Data not shown). Root-specific LUC activity can be first detected in 2 week-old, *in vitro* or soil-grown T_2 plants, indicating that expression of *AtPRB1* is under developmental control.

The organ-specific expression pattern of the *AtPRB1*::*LUC* gene fusion was quantitatively determined in independent T_2 transgenic *Arabidopsis* plants using an *in vitro* LUC assay involving luminometry measurements. In vegetative organs there was a moderate level of LUC activity in roots and stems and essentially no detectable LUC activity in mature leaves (Fig 3.6A). Specifically, the average LUC activity recorded in roots was 590 Relative Light Units (RLU) per μg of protein compared to 610 RLU per μg of protein in stem tissue. Floral organs showed higher levels of LUC activity, with an average value of 1500 RLU per μg of protein. The expression of the *AtPRB1*::*LUC* transgene was therefore congruent with the expression of the endogenous *AtPRB1* gene. Although the same overall pattern of *AtPRB1*::*LUC* expression was observed in each plant examined, there was, as expected, some quantitative variation among independent transformants. This observed variation is probably due to 'position effect' and/or transgene copy number.

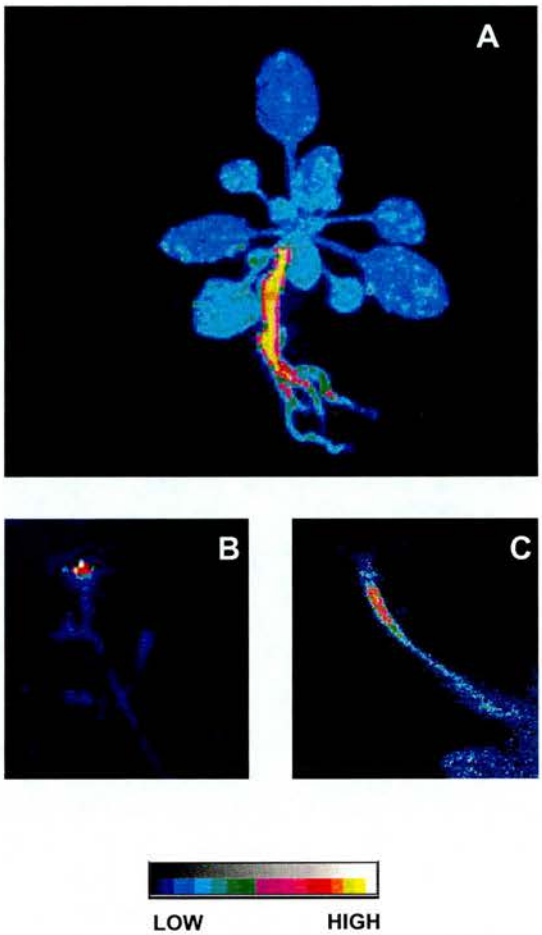


Figure 3.5. *In vivo* imaging of the pattern of LUC activity established by the *AtPRB1* promoter in a typical generated transgenic *Arabidopsis* plant. Image of LUC activity present in roots, panel (A); an *Arabidopsis* flower, panel, (B); and, a single stem, panel (C). In each case, the given organ was selectively sprayed with 1 mM luciferin and imaged twenty minutes later using an ultra low light imaging camera system.

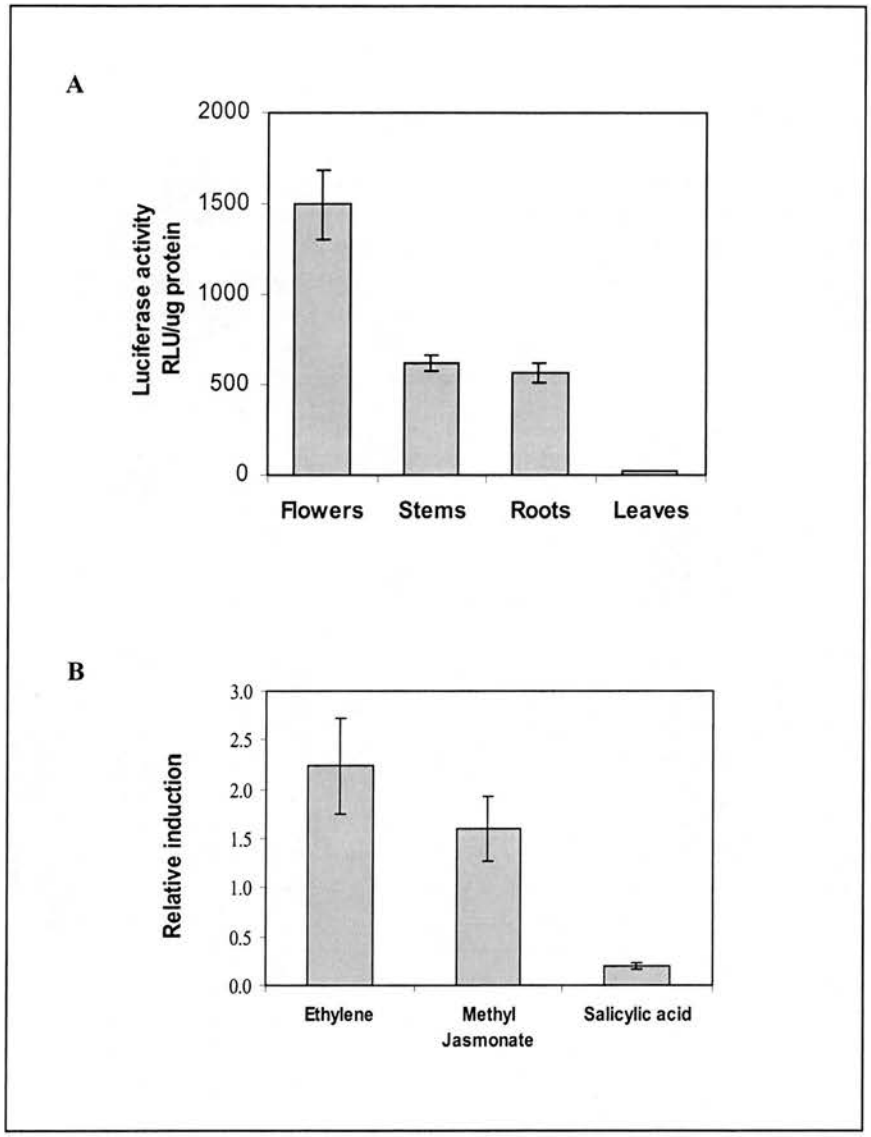


Figure 3.6. Histogram depicting the level of LUC activity established by the -2345 bp *AtPRB1* promoter in different plant organs (A); and, responsiveness of the *AtPRB1* promoter to ethylene, Me-JA and SA (B).

3.5 The *AtPRB1* promoter is responsive to ethylene and methyl-jasmonate

The organ specific expression programme established by the *AtPRB1* gene was characteristic of the expression profile elaborated by basic *PR1* genes in other plant species (Memelink *et al.*, 1990). Some of these genes are also activated in response to attempted ingress by given pathogens (Tornero *et al.*, 1997). Basic *PR1* gene expression has also been shown to be cued by the key defence signalling molecules ethylene and Me-JA, whilst being possibly inhibited in response to SA accumulation (Eyal *et al.*, 1993; Niki *et al.*, 1998). We therefore investigated the impact of these defence signals on the expression of the *AtPRB1*::*LUC* gene in distinct organs of *Arabidopsis*. The exogenous application of either the ethylene-releasing compound ethephon (Memelink *et al.*, 1987) or Me-JA resulted in the activation of the *AtPRB1*::*LUC* transgene in *Arabidopsis* roots to a value of 2.2-fold and 1.5-fold respectively above the basal expression level in this organ (Fig 3.6B). The expression of the *AtPRB1* gene was not induced by the exogenous application of ethylene or Me-JA in stem, leaf or floral tissue.

Interestingly, the exogenous application of SA strongly repressed the activity of the -2345 bp *AtPRB1* promoter in roots (Fig 3.6B). This observation supported the finding of a previous study which reported that the wound-induced accumulation of transcripts for 4 basic *PR* genes was enhanced by Me-JA but inhibited in the presence of SA (Niki *et al.*, 1998). Thus, the expression of the *Arabidopsis AtPRB1* gene is regulated in a similar fashion to many other characterised basic *PR1* genes in response to key defence signals (Brederode *et al.*, 1991; Niki *et al.*, 1998).

3.6 Impact of 5′-promoter deletion mutants on organ-specific expression

In order to identify regions of the *AtPRB1* promoter that contain key *cis*-elements necessary to establish the complex expression pattern of this gene we generated a limited series of 5′-promoter deletion mutants. Chimeric *AtPRB1*::*LUC* genes containing -989 bp and -319 bp of the *AtPRB1* promoter sequence (Fig 3.4) were transformed into *Arabidopsis* accession Col-0 to generate transgenic plants.

Independent transgenic lines were randomly selected for each *AtPRB1* promoter deletion mutant and T_2 plants were employed for further analysis. The value of LUC activity established by the -989 bp *AtPRB1* promoter deletion mutant was significantly reduced in floral organs and stems to 65% and 73% respectively of that observed for the -2345 bp *AtPRB1* promoter (Fig 3.7A, B and C). In roots however, the level of LUC activity measured was not significantly different from that produced in root tissue derived from transgenic plants containing the -2345 bp *AtPRB1*::*LUC* construct (Fig. 3.7A and D). In contrast, the -319 bp *AtPRB1* promoter deletion mutant elaborated a different expression profile: while LUC activity was reduced to 13% and 17% of that established by the -2345 bp *AtPRB1* promoter sequence in floral organs and stems (Fig 3.7A, B and C), the recorded LUC activity in roots was increased to a value of 152% (Fig 3.7A and D). In a similar fashion to the -2345 bp *AtPRB1* promoter sequence neither of these promoter deletion mutants established significant expression in leaf tissue (Fig 3.7A). Thus, sequential deletion of the *AtPRB1* promoter from -2345 bp to -989 bp to -319 bp resulted in a graded reduction in LUC activity in both floral organs and stems. However, in roots the results were strikingly different: while deletion of the *AtPRB1* promoter from -2345 bp to -989 bp had no significant impact on the level of LUC activity determined, deletion of the *AtPRB1* promoter from -989 bp to -319 bp resulted in a significant increase of LUC activity, to a value of 152% of that established by the -2345 bp *AtPRB1* promoter sequence.

3.7 Impact of 5′-promoter deletion mutants on ethylene and Me-JA responsiveness

We also analysed the response of the *AtPRB1* promoter deletion mutants in root tissue to the defence signalling molecules ET and Me-JA. The -2345 bp *AtPRB1* promoter sequence was activated 2-fold following treatment with the ethylene-releasing compound ethephon (Fig 3.8A and B). Interestingly, the levels of LUC activity established by the -989 and -319 *AtPRB1* 5′-promoter deletion mutants were strongly repressed in response to ethephon treatment (Fig 3.8A and B). These observations support recent studies suggesting that ethylene can either activate or

repress the expression of PR genes by modulating the activity of distinct *trans*-acting factors (Ohta *et al.*, 2000).

In contrast, the response of the *AtPRB1* promoter deletion mutants to Me-JA was conspicuously different to that observed with ET (Figure 3.8C and D). Thus, while the -2345 bp *AtPRB1* promoter sequence elaborated a 2-fold increase in LUC activity in response to Me-JA, the -989 bp and -319 bp promoter deletion mutants elaborated a 4.25-fold and a 3.4-fold increase in LUC activity respectively following exogenous treatment with Me-JA (Fig 3.8C and D).

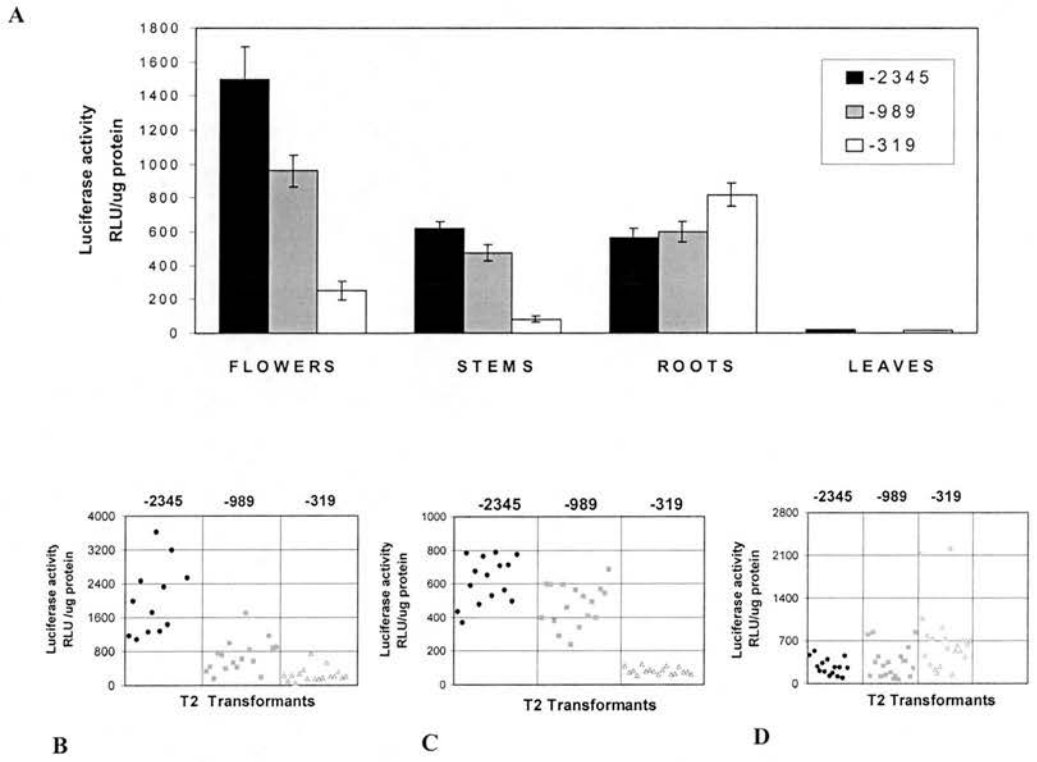


Figure 3.7. Impact of 5′-promoter deletion mutants on the organ-specific expression pattern established by the *AtPRB1* gene promoter. Histogram depicting the average value of LUC activity established for each 5′-promoter deletion mutant in flowers, stems, roots and leaf tissue (A). The level of LUC activity determined for individual T_2 transgenic plants in flowers (B), stems (C) and roots (D) is shown.

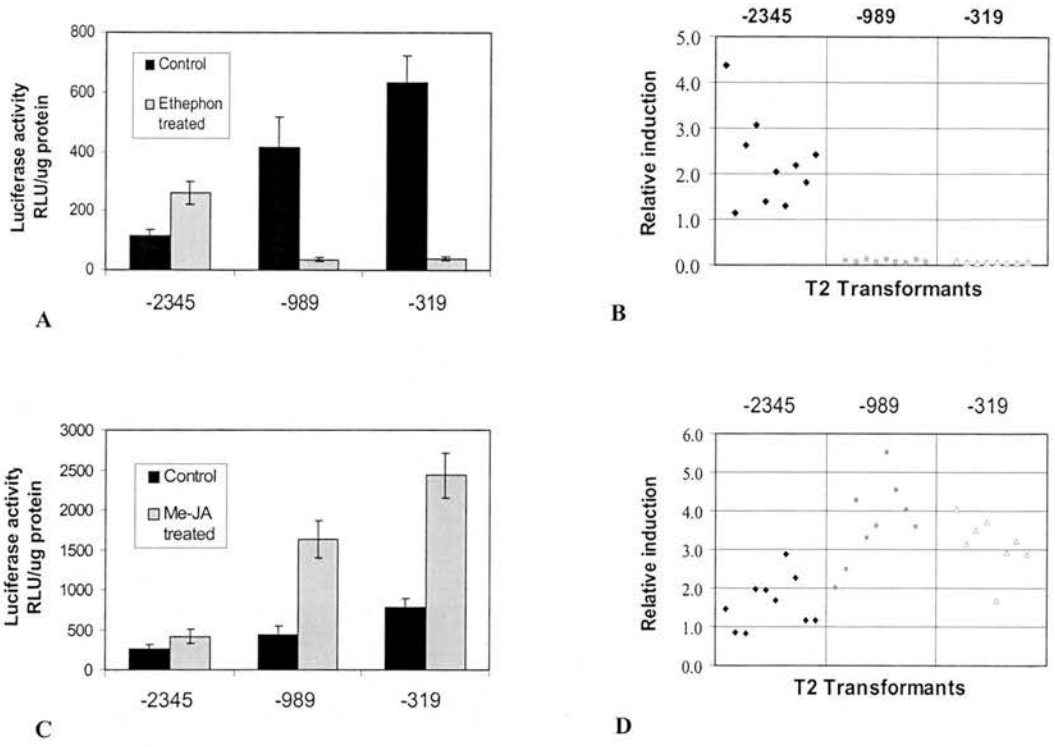


Figure 3.8. Impact of 5′-promoter deletion mutants on the responsiveness of the *AtPRB1* promoter to ET and Me-JA. Histograms illustrating the average relative induction of LUC activity for given *AtPRB1* promoter deletion mutants in response to either ethephon treatment (A) or Me-JA treatment (C) compared to the average LUC activity values for buffer treated control plants. The relative value of induction of LUC activity determined for individual T_2 transgenic plants, containing given *AtPRB1* promoter deletion mutants, that have been exogenously treated with either ethephon (B) or Me-JA (D).

3.8 Expression of AtPRB1 protein in *E.coli*

In tobacco, several PR-1 proteins were previously shown to have inhibitory activity against oomycete pathogens (Alexander *et al.*, 1993; Niderman *et al.*, 1995). The expression profile elaborated by *AtPRB1*, with roots exhibiting significant levels of expression and responsiveness to Me-JA and ET, prompted us to investigate whether its inhibitory activity could be effective against non-oomycete root pathogens. The *AtPRB1* coding region was therefore cloned into pRSET-B (Invitrogen expression system) to express and purify the putative protein and test its functional activity. The

slow growth of transformed *E.coli* BL21 (DE3) cells and small colony size relative to the control cells carrying the empty vector indicated protein toxicity (Fig 3.9B). To compare the growth kinetics between the IPTG induced and non-induced control cultures, the cells were grown in liquid medium and the cell number was quantified in a time course by spectrophotometry (Fig 3.9A). At five hours following inoculation, the cell density in the IPTG (1mM) induced culture was approximately 8-fold less than that of the control cells containing the empty vector. Attempts to express the protein under modified conditions such as reduced IPTG concentration (0.5mM), low incubation temperature (25°C) and denaturing buffers, failed to improve the protein recovery (as observed in SDS-PAGE). While these results suggest that growth inhibition is caused by the AtPRB1 protein, the direct anti-microbial activity remains to be rigorously established.

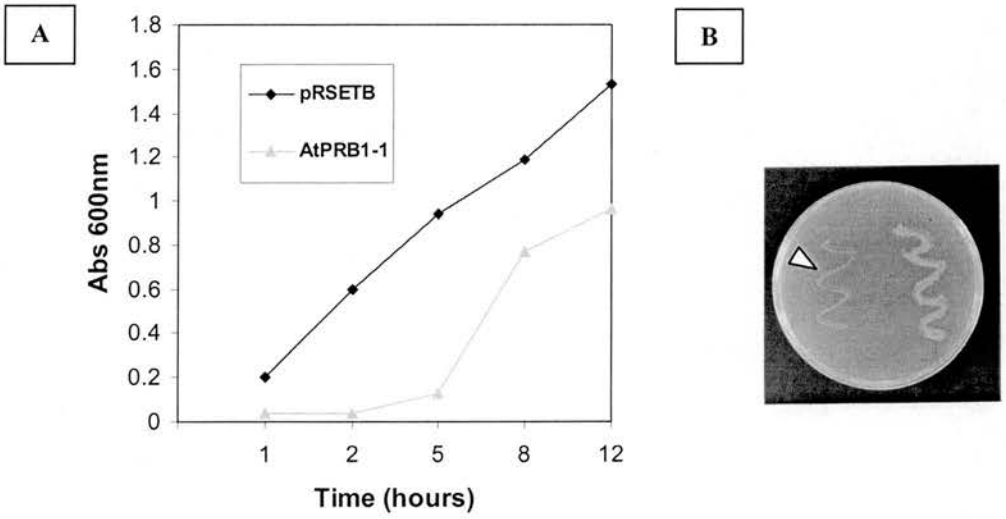


Figure 3.9. AtPRB1 protein expression in *E.coli* BL21(DE3) (A) Growth kinetics of *E.coli* BL21(DE3) expressing a recombinant AtPRB1 protein (grey) compared to bacterial cells carrying the empty pRSETB (black). (B) Cells were grown in LB media. Arrowhead indicates recombinant colonies, control colonies are on the right hand side of the petri dish.

CHAPTER 4

4.1 Discussion

A genomic fragment containing 5′-regulatory sequences of the *PR1*-like gene, *AtPRB1*, has been isolated and fused to the gene encoding the LUC of *P.pyralis* and the resulting chimeric construct transformed into Col-0 *Arabidopsis* plants. The *AtPRB1* promoter established an exquisite organ-specific expression programme. In this context, LUC activity was observed in floral organs, stems and roots. The expression programme mediated by the *Arabidopsis AtPRB1* promoter therefore resembled that observed for several other basic *PR1* genes in dicots including tobacco (Memelink *et al.*, 1990; Eyal *et al.*, 1993). Moreover, a similar organ-specific expression pattern for a basic PR gene has also been reported in monocotyledoneous rice plants (Xu *et al.*, 1996). In *N. tabacum* for example, the promoter of a basic *PR1* gene drove the expression of a β-glucuronidase (GUS) reporter gene in floral organs, roots and stems (Eyal *et al.*, 1993). Interestingly, histochemical studies of these transgenic plants revealed that GUS expression in stems was restricted to the vascular tissue. In contrast, the promoter sequence of a basic *PR1* gene from tomato failed to drive constitutive expression of a GUS reporter gene in any vegetative tissues examined (Tornero *et al.*, 1997), providing a notable exception to the characteristic expression pattern elaborated by basic *PR1* gene promoters. The organ-specific expression programme established by the basic *Arabidopsis AtPRB1* gene described here therefore closely resembles that elaborated by many other basic *PR* genes (Eyal *et al.*, 1993; Memelink *et al.*, 1990; Xu *et al.*, 1996). Thus, the regulatory networks establishing the organ-specific expression of this class of genes appears to be conserved in *Arabidopsis*. The exquisite organ-specific expression programme established by the *AtPRB1* gene promoter suggests AtPRB1 may undertake important functions beyond a possible role in disease resistance. Indeed, the basic tobacco PR-2d has been proposed to play an integral role in seed germination (Vögeli-Lange *et al.*, 1994), while PR3- and PR4-like proteins are thought to function as morphogenetic factors in carrot embryogenesis (De Jong *et al.*, 1992). In addition, a correlation between plant senescence and the

accumulation of defence-related proteins has been well established (Quirino *et al.*, 1999). Ethylene treatment can induce the expression of *PR2* (Gheysen *et al.*, 1990) and *PR3* (Melchers *et al.*, 1993) in tobacco plants. This plant hormone has also been implicated in the regulation of fruit ripening, flower and leaf senescence, and leaf abcission (Abeles *et al.*, 1992). Transgenic plants showing low ethylene levels and ethylene-insensitive mutants both exhibit delayed leaf senescence (Grbic and Bleecker, 1995). The production of defence proteins could contribute to enhance the defensive capacity of the plant during the critical time of flowering and seed formation and protect the senescing leaves from invasion by opportunistic pathogens (Morris *et al.*, 2000). However, it is also possible that the induction of defence related proteins could be an integral part of the senescence mechanism. A role in the redistribution of nutrients and cellular disruption could be envisaged for those proteins that have hydrolytic activity on particular plant substrates (Obregon *et al.*, 2001).

The generation and subsequent analysis of a nested series of 5'-deletion mutants of the *Arabidopsis AtPRB1* promoter identified regions responsible for mediating the expression pattern of this gene. Deleting the -2345 bp promoter to -989 bp and -319 bp reduced constitutive LUC activity in floral organs to 65% and 13% respectively of that recorded for the -2345 bp promoter. A similar observation was obtained for stem specific expression. In this case, the -989 and -319 bp promoter mutants established reduced LUC activity to a level of 73% and 17% of that observed for the -2345 bp promoter. Thus, positive quantitative elements which drive the constitutive expression of the *AtPRB1* gene in floral organs and/or stem tissue reside between -2345 bp to -989 bp and -989 bp to -319 bp. In this context, two G-box core motifs (ACGT, -2120 to -2117 and -1015 to -1018) (Foster *et al.*, 1994) are located between -2345bp and -989 bp, and a further G-box core motif is found between 989 bp to -319 bp (-942 to -939). This motif has previously been shown to convey the capacity for flower and stem specific expression onto a minimal chalcone synthase gene promoter in transgenic tobacco (Faktor *et al.*, 1997).

The impact of these *AtPRB1* promoter deletion mutants on LUC activity in roots was distinct from that observed in floral organs and stems. Thus, while deletion of the *AtPRB1* promoter to -989 bp did not significantly impact the level of LUC activity measured in roots, the -319 bp *AtPRB1* promoter deletion mutant elaborated increased LUC activity in this organ. Hence in roots, a *cis*-element which resides between -989 bp and -319 bp may bind a negative regulator of root specific expression. A prominent feature of this region is the presence of a motif (ATATTT, -600 to -595) that may be required for the expression of a wheat peroxidase gene in root tissue (Hertig *et al.*, 1991). Moreover, this region contained four copies of the core sequence of the Box I and II binding sites for the transcription silencing factor SBF-1 (Harrison *et al.*, 1991). The presence of these motifs significantly reduced the expression of a bean CHS15 gene in suspension cells derived from soybean root tissue (Dron *et al.*, 1988). Thus, the removal of potential SBF-1 binding sites in the -989 bp to -319 bp deletion may be a significant factor in the corresponding increase in *AtPRB1*::*LUC* expression observed in this organ.

Superimposed upon the organ-specific expression pattern established by the *Arabidopsis AtPRB1* promoter was the engagement of *AtPRB1* gene expression in root tissue following the exogenous application of either ET or Me-JA, which are both key cues for the deployment of plant defence responses (Pieterse *et al.*, 1998; Thomma *et al.*,1998). The response of the *AtPRB1* promoter to ET again resembled that observed for other basic *PR1* genes, which have been reported to be induced by this hormone (Memelink *et al.*, 1990; Eyal *et al.*, 1992; Brederode *et al.*, 1991; Ohtsubo *et al.*, 1999). Moreover, recent studies have also shown the activation of a basic *PR1* gene in response to Me-JA (Niki *et al.*, 1998). Hence, the activation of the *Arabidopsis AtPRB1* promoter in response to these key defence cues also correlates closely to that previously observed with other basic *PR1* genes. The defence signalling circuitry mediating the expression of many basic *PR1* genes in other plant species may therefore be conserved in *Arabidopsis*.

The deployment of nested 5′-deletion mutants of the *Arabidopsis AtPRB1* promoter identified regions responsible for mediating the expression pattern of this gene in

response to ET and Me-JA. The -2345 bp *AtPRB1* promoter was induced 2-fold following the exogenous application of the ET releasing compound ethephon to root tissue. In contrast, deletion of the *AtPRB1* promoter to either -989 bp or -319 bp resulted in a strong repression of LUC activity in response to ethephon treatment. Thus, the cognate *trans*-factor of a *cis*-element located between -2345 bp and -989 bp of the *AtPRB1* promoter may engage *AtPRB1* gene expression in response to ET accumulation. This region contains a near perfect GCC box (AGCCACC, -1997 to -1991) which is a characteristic feature of many ET inducible promoters and has been shown to be involved in the ET mediated activation of a *PR-5* gene in tobacco (Sato *et al.*, 1996). Moreover, this element is located close to a G-box core motif, a similar arrangement to that found in the ethylene responsive *PDF1.2* gene (Manners *et al.*, 1998). ET has also recently been shown to repress gene expression via the GCC box. This apparent paradox may be explained by the action of distinct ethylene-responsive factors (ERFs) which bind this motif. Thus, while ERF-2 and -4 mediated transcriptional activation, ERF-3 binding resulted in strong repression (Ohta *et al.*, 2000). No GCC box motifs were identified in the *AtPRB1* promoter below -989 bp. Hence, the ET mediated repression of LUC activity observed with the -989 bp and -319 bp *AtPRB1* promoter deletion mutants may therefore be mediated via an alternative *cis*-element. Another potential regulatory sequence identified in the region between -2345 bp and -989 bp is a W box type element ((T)TGAC(C); -1588 to –1594, -1612 to –1617, -1632 to –1637 and –1657 to –1662) present in a large number of *PR* gene promoters (Rushton and Somssich, 1998). The WRKY proteins are a superfamily of transcription factors with a binding preference for the W box. WRKY proteins were induced in cultured parsley cells in response to fungal elicitor treatment and were able to bind specifically *in vitro* to W box promoter elements (Rushton *et al.*, 1996). W boxes frequently cluster within short promoter stretches and can act together synergistically (Eulgem *et al.*, 1999). Moreover, a palindromic arrangement of W box motifs was shown to confer more rapid transcriptional activation. Additional elements including the GCC box have been proposed to participate in WRKY- mediated activation (Eulgem *et al.*, 1999). This arrangement occurs in the *AtPRB1* promoter, in which the palindromic sequence consists of a core W box motif (TTGACC, -1588 to –1594) located beside three reversely oriented

motifs (GGTCAA, -1612 to –1617, -1632 to –1637 and –1657 to –1662) and a nearby GCC box element (-1991 to –1997), suggesting that the W boxes are functional. In the *Arabidopsis PR-1* gene, the basal and SA-induced expression levels are thought to be negatively regulated by W boxes (Lebel *et al.*, 1998). As the exogenous addition of SA was also found to repress *AtPRB1* promoter activity, it seems possible that the W-box may be involved in SA-mediated regulation.

The impact of 5′-deletion analysis of the *AtPRB1* promoter with respect to Me-JA responsiveness was strikingly different to that observed for ET. Thus, while the -2345 bp *AtPRB1* promoter elaborated a 2-fold increase in LUC activity in response to exogenous application of Me-JA to root tissue, the -989 bp and -319 bp deletion mutants showed increased responsiveness to this cue, producing a 4.25-fold and 3.3-fold increase in LUC activity respectively. Therefore, in the context of responsiveness to Me-JA, a negative regulatory element may be situated between -2345 bp and -989 bp of the *AtPRB1* promoter while a positive regulatory element may reside between -989 bp and -319 bp. Interestingly, in the latter region, two copies of a sequence (AATGTT, -899 to -894 and -776 to -771) found in the Me-JA responsive regions of a soybean *VspB* gene (Mason *et al.*, 1993) and barley lipoxygenase genes (Rouster *et al.*, 1997) were uncovered. Furthermore, this sequence was also found in the Me-JA responsive *Arabidopsis* defence gene *PDF1.2* (Manners *et al.*, 1998). Further work, including the generation of a fine series of 5′-promoter deletion mutants in combination with linker-scanning and/or site directed mutagenesis will be required to precisely define given *cis*-elements within the *AtPRB1* promoter that convey responsiveness to organ-specific and environmental cues.

Interestingly, the expression of the *AtPRB1*::*LUC* gene was strongly suppressed in response to SA, a key metabolite required for the development of systemic acquired resistance (Malamy *et al.*, 1990). This observation is consistent with the discovery that basic PR-proteins do not accumulate in tobacco foliar tissue exhibiting SAR (Brederode *et al.*, 1991). Thus, AtPRB1 is unlikely to contribute to the development of this defence mechanism. Indeed, the exogenous application of SA has been shown

to inhibit the accumulation of some basic PR1 proteins in tobacco in response to endogenous Me-JA (Niki *et al.*, 1998). Other examples of cross-talk between the Me-JA-dependent and SA-dependent defence signalling pathways have also now begun to emerge. Addition of exogenous di-iso-nicotinic acid, a potent inducer of SAR, suppressed the constitutive expression of the Me-JA responsive *PDF1.2* gene in the *Arabidopsis cpr5* mutant (Bowling *et al.*, 1997). Moreover, an inverse relationship has recently been demonstrated between the development of SAR against microorganisms via an SA-dependent mechanism and resistance to insect herbivory mediated via the accumulation of Me-JA (Felton *et al.*, 1999). In this context, distinct Me-JA and SA-dependent defence response networks are also thought to be essential for resistance against given microbial pathogens. Thus, a SA-dependent network is thought to mediate resistance against biotrophic pathogens such as *Peronospora parasitica*, whilst a Me-JA dependent network is thought to engage resistance against necrotrophic pathogens such as *Alternaria brassicicola* (Thomma *et al.*, 1998).

While the constitutive expression of the *AtPRB1* gene in roots, stems and flowers may contribute to the establishment of horizontal resistance in these organs, the activation of the *AtPRB1* gene promoter by Me-JA and ET and its repression by SA in root tissue, suggests this gene may be important for the establishment of resistance against necrotrophic pathogens. In addition, the exquisite organ-specific expression programme established by the *AtPRB1* gene promoter suggests AtPRB1 may undertake important functions beyond a possible role in disease resistance. We anticipate that transgenic plants containing the *AtPRB1* based promoter-reporter constructs will provide useful tools for the future dissection of the signalling networks regulating the expression of the *AtPRB1* gene.

Expression of the AtPRB1 protein in *E.coli* resulted in inhibition of bacterial growth. The cell growth of *E.coli* containing the cloned *AtPRB1* gene was approximately 8-fold less than that of the cells containing the empty vector. Inhibition occurred regardless of induction by IPTG. These results suggest that inhibition was caused by the AtPRB1 protein but we have not yet established whether this is a direct consequence of AtPRB1 activity. A different approach such as *in vitro* translation

may provide an alternative method for the purification of AtPRB1. Once the protein purification is achieved, it will be possible to test the activity of AtPRB1 against root necrotrophic pathogens and therefore validate our hypothesis. In support of this idea is the observation in tobacco ET-insensitive mutants, that ET perception is required for basic PR-gene expression and resistance against the root pathogen *Pythium sylvaticum* (Knoester *et al.*, 1998).

CHAPTER 5

Identification of *Arabidopsis* SAR mutants by Luciferase imaging

5.1 Introduction

Resistance to pathogens is controlled by complex signal transduction networks (Genoud *et al.*, 2001). Plants are able to differentially activate distinct defence pathways depending on the pathogen they encounter. The existence of multiple defence mechanisms is possibly an evolutionary adaptation to challenge from different groups of pathogens (Thomma *et al.*, 2001). As discussed previously in the general introduction, the signalling molecules SA, JA and ET play key roles in the activation of these defence pathways. Mutant screens in *Arabidopsis* have identified a considerable number of genes involved in disease resistance (reviewed by Feys and Parker, 2000). Many of these mutants uncovered components of the SA-dependent signalling network, providing conceptual models to understand the defence responses underlying SAR. Towards this end, a transgenic *Arabidopsis* line was created that contains a *PR-1*::*LUC* promoter-reporter fusion (Murray *et al.*, unpublished data). Because *PR-1* gene induction is a reliable molecular marker for the establishment of SAR in tobacco and *Arabidopsis*, we utilised this genetic background to undertake a screen for an altered SAR response. Initially, a population of 5000 mutant lines was generated in this designer background by activation tagging. The primary screen involved imaging for constitutive LUC activity or absence of LUC activity following induction with the SA analogue BTH. We sought to isolate mutants displaying enhanced disease resistance as well as enhanced disease susceptibility.

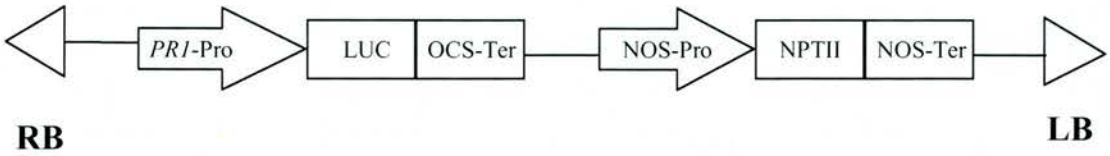
Generation of mutant phenotypes by activation tagging is based on gene activation by transcriptional enhancers. In this system, four CaMV 35S enhancer repeats located near the border of a T-DNA can cause ectopic expression from promoters of immediately adjacent genes, resulting in dominant phenotypes. It was the method chosen to produce the mutant lines because it can potentially identify genes with either redundant or essential functions not easily identifiable by loss of function screens. If the mutation is the result of gene activation, the tagging vector can be

used for the rapid cloning of the mutated gene. In addition, loss of function mutants can be generated by gene disruption.

5.2 Generation of activation-tagged *Arabidopsis* lines

A transgenic *Arabidopsis* ecotype Col-0 line containing a chimeric *PR-1*::*LUC* gene fusion (Fig 5.1A) had been successfully used to determine the temporal and spatial expression of the *PR-1* gene during the establishment of local and systemic resistance (Murray *et al.*, unpublished data). The induction of the endogenous *PR-1* gene following *Pst* DC3000 (*avrB*) inoculation was also found to be consistent with induction of the *PR-1*::*LUC* transgene, confirming that it functions as an accurate reporter (Murray et al., unpublished data). This *Arabidopsis PR-1*::*LUC* line was used to generate activation-tagged mutants and screen for an altered *PR-1*::*LUC* response. The *Arabidopsis* plants were transformed *via Agrobacterium* with the activation-tagging binary vector SKI015 (Walden *et al.*, 1994; Weigel *et al.*, 2000) (Fig 5.1B). Integrated into the transformation vector is the bar gene, which confers resistance to the herbicide BASTA® (glufosinate) and is useful for high-throughput selection of transgenic plants on soil. Primary transformants (T_1) were thus selected on soil for BASTA resistance. Following this selection, plants were allowed to self-fertilise and seeds were harvested from each T_1 plant individually. A total of 5000 independent lines were then screened by luciferase imaging.

A



B

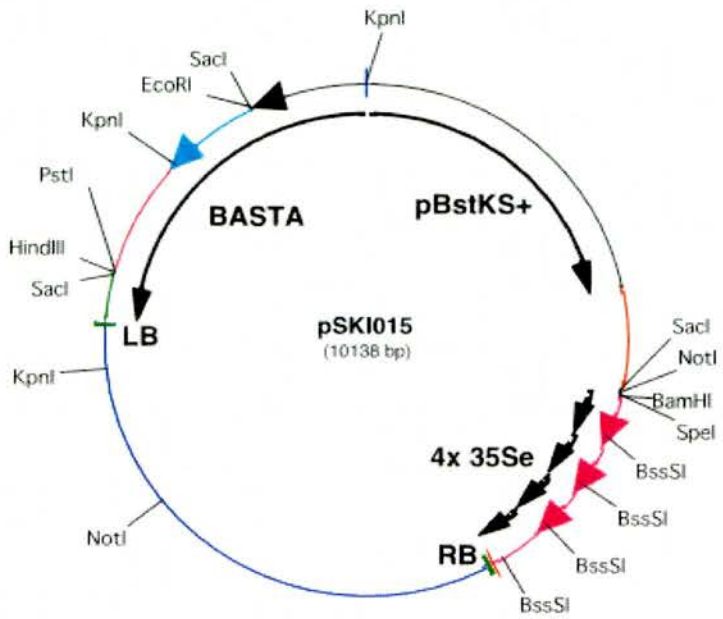


Figure 5.1. A) Transformation cassette used to create the *PR-1*::*LUC* transgenic line. LB-left border, LUC-luciferase gene, NPTII- neomycin phosphotransferase (encoding kanamycin resistance), NOS- nopaline synthase, OCS- octopine synthase, Pro- promoter, RB- right border, Ter-terminator. **B) Activation tagging vector pSKI015.** T-DNA insertion cassette enclosed by LB-left border and RB-right border; containing BASTA resistance gene, origin of replication (BluescriptSK$^+$), and tetramer of CaMV 35S enhancer (Walden *et al.*, 1994; Weigel *et al.*, 2000).

5.3 Isolation of candidate SAR mutants

Initial experiments on agar plates had indicated that seedlings grown on MS media showed high background LUC activity resulting from *in vitro* growth conditions. This approach was also time-consuming and laborious and therefore it was decided to continue the screen on soil-grown plants under optimised conditions. Two different screens were conducted on identical sets of activation-tagged T_2 lines. For simplification purposes the screen for mutants that constitutively express *PR-1::LUC* without induction is referred to as constitutive PR-1 expressor screen and the screen for mutants that do not express *PR-1::LUC* after induction is referred to as a suppressor screen.

5.3.1 Constitutive PR-1 expressor screen

T_2 seedlings were grown on soil for 10 days before the leaves were painted with luciferin and imaged under the ultra low-light camera for constitutive LUC activity. A dominant mutation resulting from an activated gene was expected to show a 3:1 segregation ratio in the T_2 generation. Alternatively, loss-of-function mutations produced by gene disruption were expected to segregate as a recessive trait. In this case the segregation ratio would be 1:3. In order to isolate both types of mutations, candidate mutants were selected for LUC expression above the background level, in at least 25% of the seedlings from a single line. An example of a constitutive PR-1 candidate mutant identified in the primary screen is shown in Fig 5.2A. Candidate mutants were allowed to self-fertilise and the T_3 seed was collected for further screening and confirmation of the mutant status. A set of 34 candidate mutants were re-tested and from this initial group, a subset of 5 putative mutants consistently showed LUC activity in the secondary screen (Table 5.1). They were subsequently named *esr1* to *esr5* (for enhanced systemic resistance) and further characterised.

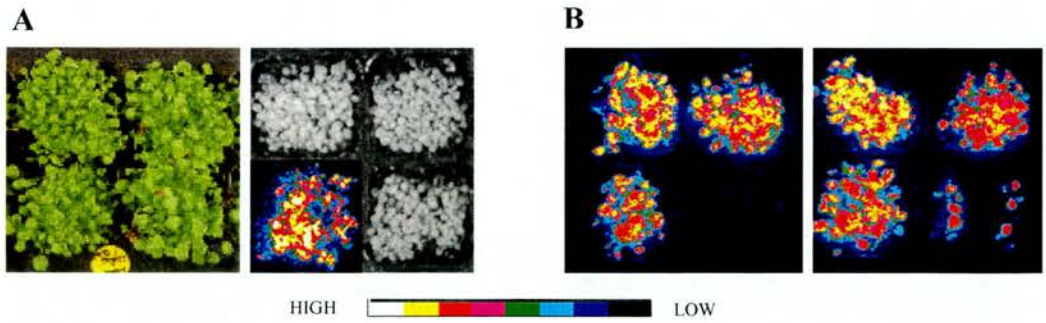


Figure 5.2. Representative examples of a constitutive PR-1 candidate mutant (A) and two categories of suppressor candidate mutants (B).

A) Photograph showing 10-day old seedlings from four independent lines (left panel) and bright-field image showing constitutive LUC activity in one of the four lines (right panel).
B) Leaves were sprayed with 1 mM BTH and imaged after 5 days. LUC activity is absent in all seedlings corresponding to one of the four lines shown (left panel). No more than ~25% of seedlings exhibit LUC activity in one of the four lines shown (right panel).

5.3.2 Suppressor screen

The suppressor screen consisted of spraying 7-day old seedlings with BTH (1mM) and imaging after 5 days for no LUC activity following BTH induction. Previous studies had shown that BTH-induced *PR-1* expression peaks approximately 5 days after treatment. They also showed that BTH is a more powerful inducer of SA-dependent responses than SA and it offers the additional advantage of lower toxicity to plants (Lawton *et al.*, 1996). This strategy allows for the identification of mutations resulting from an activated gene that can block SA-dependent pathways. Different types of candidate suppressor mutants were isolated in the primary screen. They can be described in two categories: (1) absence of LUC activity in 100% of seedlings corresponding to an individual line; and (2) segregation of LUC activity among the seedlings corresponding to an individual line. Representative examples of both categories are presented in Fig 5.2B. The expected segregation ratio for a dominant mutation was 3:1 and therefore lines exhibiting LUC activity in no more

than 25% of the seedlings corresponding to a single line were selected as candidate suppressor mutants. There was a possibility that the suppressor candidate mutants did not show induction of LUC activity following BTH treatment because the *PR-1::LUC* transformation cassette had been eliminated or rearranged in the plants. In order to verify that the candidate mutant lines contained an intact transformation cassette, seeds were selected on MS medium containing kanamycin. Only lines showing 100% kanamycin resistance were further tested in the secondary screen. A total of 162 candidate mutants were isolated in the primary screen. Among these, 121 T_3 putative mutants were later confirmed in the secondary screen (Table 5.1).

	Total No. of lines screened	**No. of putants Primary screen**	**No. of putants Secondary screen**
Constitutive PR-1 expressor screen	5000	34	5
Suppressor screen	5000	162	121

Table 5.1. Number of putative constitutive PR-1 expressor and suppressor mutants isolated in the primary and secondary screens.

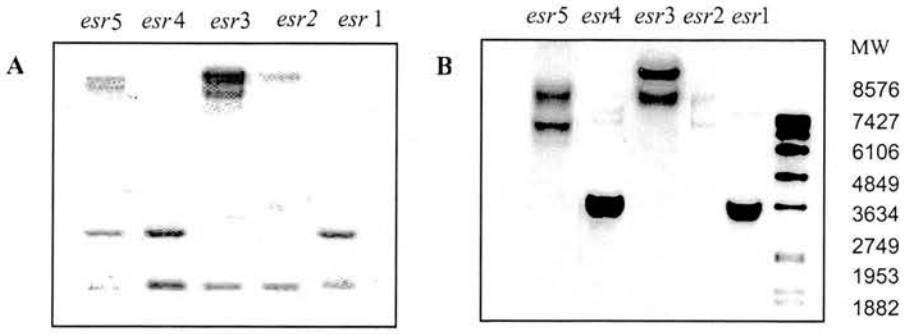
5.3.3 BASTA segregation and Southern blot analysis

The segregation of BASTA resistance and the co-segregation of the mutant phenotype with BASTA resistance were investigated in the five constitutive PR-1 putative mutants. T_2 plants were first phenotypically scored by imaging for constitutive LUC activity/no LUC activity and then sprayed with the BASTA solution. The segregation ratio of BASTA resistant to sensitive plants and the correlation to *PR-1::LUC* expression are recorded in Table 5.2 .

LINE No.	No. OF T2 PLANTS TESTED	BASTA SEG (res:sen)	χ^2 (3 : 1)	*PR1::LUC* SEG (exp:no exp)	χ^2 (1 : 3) r	χ^2 (3 : 1) d
esr 1	56	41:15	0.76	41:15	7.9E-17	0.76
esr 2	68	0:68	2.8E-46	60:8	2.1E-33	0.012
esr 3	93	22:71	2.8E-30	26:67	0.52	1.3E-25
esr 4	41	32:9	0.65	31:10	8.9E-14	0.92
esr 5	51	47:4	4.7E-03	36:15	6.6E-14	0.47

Table 5.2. Segregation of the *esr* mutations in the T_2 progeny from primary transformants. BASTA segregation is expressed as ratio of resistant to sensitive plants. *PR-1::LUC* segregation is expressed as ratio of *PR-1::LUC* expressing to non-expressing plants. The observed segregation was compared to the expected ratios for a dominant (3:1) or a recessive trait (1:3) using the *chi*-squared test. Significant χ^2 probability values are underlined ($P < 0.1$ rejects hypothesis).

Only two mutant lines, *esr1* and *esr4*, showed a 3:1 (resistant:sensitive) segregation ratio, indicating a single insertion. Both mutant lines also showed co-segregation of *PR-1::LUC* expression and BASTA resistance indicating the presence of a tagged mutation. To ascertain the number of T-DNA insertions in the remaining mutant lines, Southern hybridisation was carried out using two vector probes (Fig 5.3). The number and size of the bands corresponding to each *esr* mutant was not consistent in the two hybridisation experiments, suggesting that the Bluescript probe is unspecific. Further Southern analysis using different probes and restriction enzymes may help to clarify this result.



Mutant	Bluescript probe	CaMV35S Enhancer probe
	No. of copies	
esr1	2	1
esr2	2	0
esr3	4	3
esr4	2	1
esr5	3	2

Figure 5.3. Southern blot analysis of putative *esr* mutants. Each lane represents DNA of *esr* mutant lines, extracted from at least two plants displaying the mutant phenotype. DNA samples were digested with *Eco* RI. Two probes were sequentially used for hybridisation, a Bluescript probe (A) and a CaMV 35S enhancer probe (B). The number of bands corresponding to each *esr* mutant are shown in the table. A shorter exposure time revealed three bands for *esr3* in B) (result not shown). Col-0 wild type and *PR-1*::*LUC* control plants included in the experiment were removed from the figure for simplification.

5.4 Phenotypic characterisation

5.4.1 Luciferase imaging and luminometer assays

The five putative mutants identified in the constitutive PR-1 screen were selected for extensive analysis of *PR-1*::*LUC* expression. A typical image of constitutive LUC activity displayed by *esr* mutants is shown in Fig 5.4.

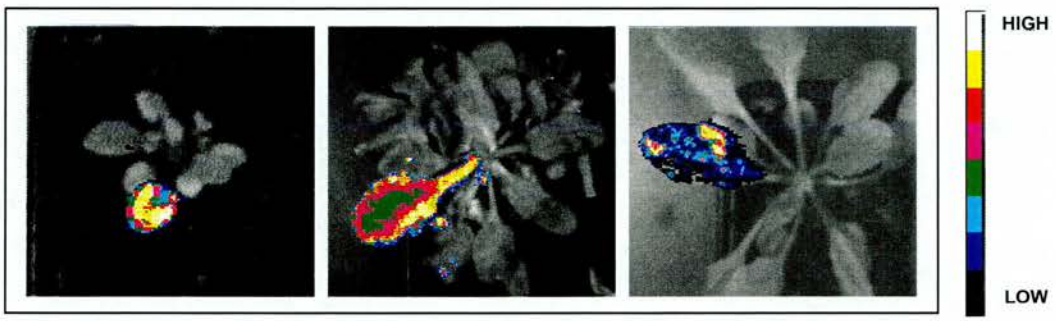


Figure 5.4. Bioluminescent images showing constitutive expression of the *PR-1*::*LUC* promoter-reporter gene in *esr* mutants. A) *esr2* B) *esr3* C) *esr5*. Four-week old plants grown in soil were imaged. One leaf from each plant was painted with 1mM luciferin and images were collected for 10s in the ultra low-light imaging camera.

The LUC activity in leaves was quantitatively determined in independent T_2 mutant plants using a LUC assay involving luminometry measurements. Fig 5.5A represents the results from a LUC assay of four-week old plants, for all candidate mutants. Untreated *PR-1*::*LUC* plants (parental transgenic line) and *PR-1*::*LUC* plants sprayed with 600µM BTH 5 days prior to the luminometer assay were also included. As expected, *PR-1*::*LUC* plants treated with BTH exhibited an increase in LUC activity relative to untreated plants. All five mutants showed increased LUC activity when compared to the parental untreated line, ranging from 15-fold in the case of *esr3* to more than 40-fold in the case of *esr2*. Only *esr2* expressed LUC activity to levels comparable to the BTH-treated *PR-1*::*LUC* line. However, the standard deviation for the average value of LUC activity in *esr2* was found to be extremely high, indicating significant variation in *PR-1*::*LUC* expression. This result was not surprising since

imaging experiments had shown that the four associated phenotypes within *esr2* exhibited different levels of LUC activity (Fig 5.6). To investigate the extent of variation in *PR-1*::*LUC* expression, a luminometer assay was performed on the four distinct *esr2* phenotypic classes, informally named *esr2-dwarf* (*d*), *esr2-yellow* (*y*), *esr2-lesions*(*l*) *and esr2-wild type-like* (*wt-l*) (Fig 5.5B). Two of these phenotypic classes, namely *esr2-d* and *esr2-y* showed levels of LUC activity several times greater than that of BTH induced plants.

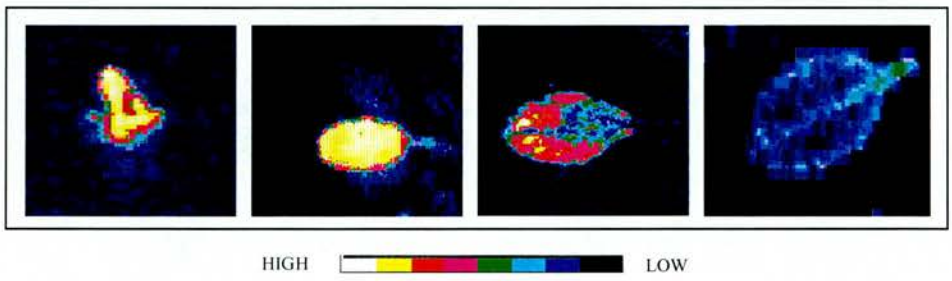


Figure 5.6. Level of LUC activity in four phenotypic classes associated with *esr2*. A) *esr2-dwarf* B) *esr2-yellow* C) *esr2-lesions* D) *esr2-wild type-like*.

In the process of imaging the candidate mutants it was also observed that levels of LUC activity in *esr3* plants varied at different stages of development. To quantify the observed increase in LUC activity over time, a luminometer assay was performed on 3-week old *esr3* plants and it was repeated two weeks later on the same group of 5-week old plants (Fig 5.5C). Remarkably, the amount of LUC activity was found to be more than 30 times higher in 5-week old plants.

The luminometer results presented in Fig 5.5 confirm the results obtained by imaging and suggest that candidate mutants are constitutively expressing *PR-1*::*LUC* and thus may define mutations in the SAR signal transduction pathway. Furthermore, the luminometer results show different levels of LUC activity for each *esr* mutant, with *esr2* and *esr3* displaying the highest LUC activity levels. The question of whether LUC activity correlates to endogenous *PR1* expression and enhanced resistance to pathogens will be addressed in the next sections.

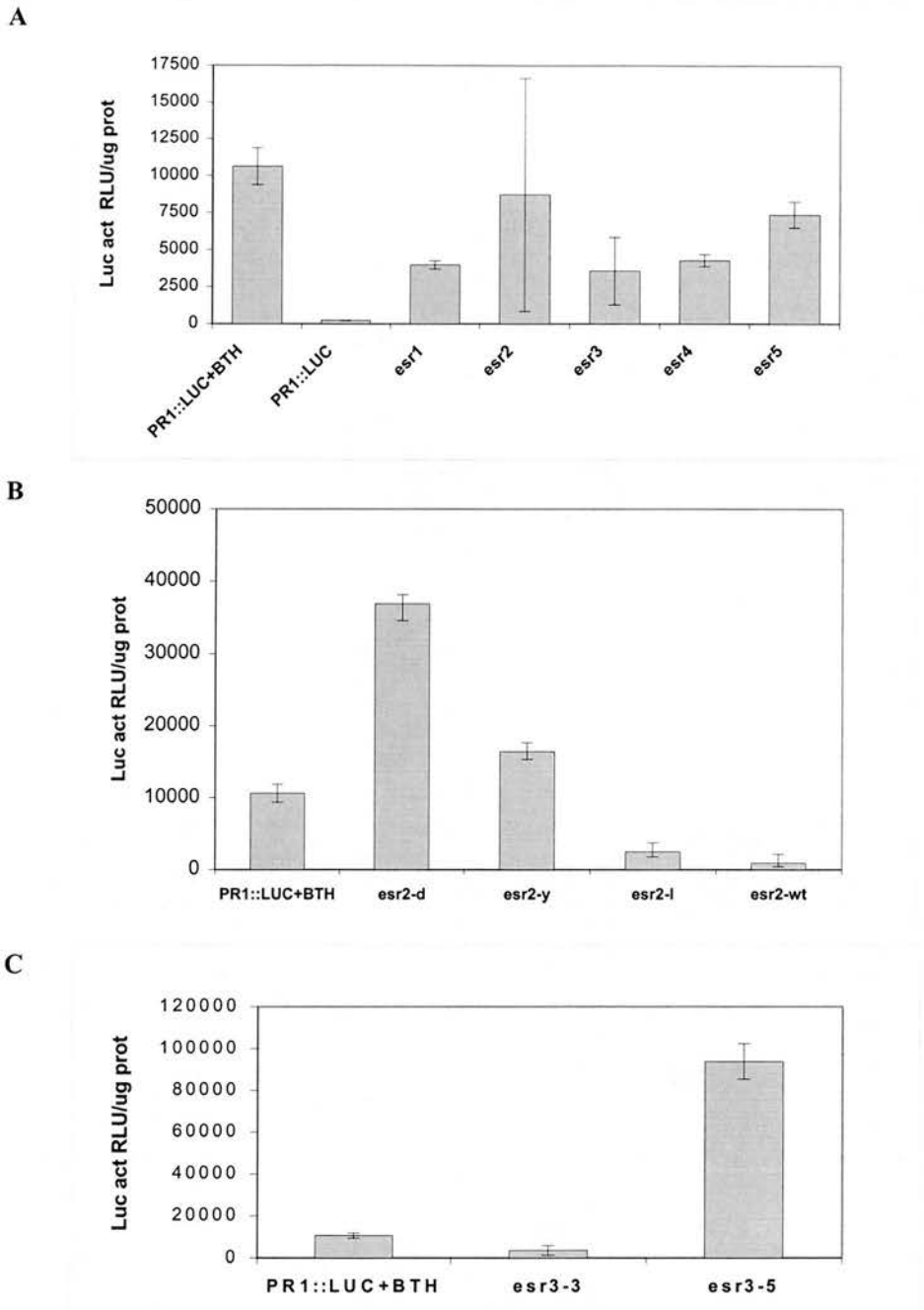


Figure 5.5. Luminometer assay of *esr* mutant lines (A), Luciferase activity of *esr2* phenotypic classes (B), Luciferase activity of *esr3* at different developmental stages (C). *Esr3*-3 and *esr3*-5 in graph (C) denote measurements taken from 3-week old plants and 5-week old plants, respectively. Each graph includes BTH treated control plants. The values represent the average of readings from at least five plants. Standard error was calculated at the 95% confidence level.

5.4.2 Northern analysis

Expression of the endogenous *Arabidopsis PR-1* gene (Uknes *et al.*, 1992) was determined in the five *esr* candidate mutants to confirm that constitutive LUC activity reflected an increase in endogenous *PR-1* gene expression. Northern blot analysis indicated that three mutants constitutively expressed the *PR-1* gene (Fig 5.7). However, no *PR-1* transcript was detected in *esr1* and *esr4*. As expected, untreated wild type plants did not show expression of *PR-1* but this gene was strongly induced in BTH-treated plants. The absence of endogenous *PR-1* expression in *esr1* and *esr4* suggested that the activation of *PR-1*::*LUC* expression in these two lines resulted from a mutation in the transgene rather than a mutation affecting endogenous gene expression. Hence, only *esr2*, *esr3* and *esr5* can be considered *bona fide* SAR mutants. *PR-1* expression was strongest in the *esr2* phenotypic classes with the exception of the wild type-like plants segregating within this line. Indeed, no *PR-1* expression was detected for this phenotypic class, confirming it is equivalent to wild type in terms of *PR-1* gene expression.

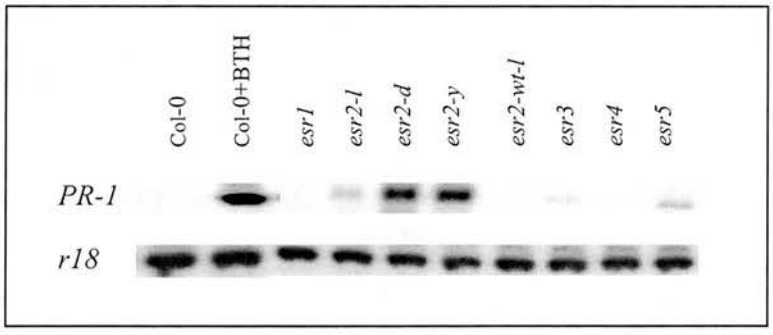


Figure 5.7. Northern blot analysis of *PR-1* gene expression in *esr* mutant lines. Each sample contains total RNA isolated from two or three plants displaying the mutant phenotype. Constitutive *r18* expression was used as loading control.

The levels of *PR-1* expression in *esr2*, *esr3* and *esr5* determined by Northern blot analysis, therefore reiterated the results obtained from the luminometer assays. Moreover, a correlation can be established between *PR-1* expression and the degree of stunting observed for each mutant. This point will be discussed more thoroughly in the section relating to 'morphological phenotype'.

5.4.3 Pathogenicity assays

Constitutive expression of the *PR-1* gene suggested that three mutant lines may have enhanced resistance to pathogen infection. To determine the level of resistance conveyed by the *esr* mutations, growth of the virulent bacterial pathogen *Pst* DC3000 and the oomycete pathogen *P.parasitica* Noco2 was assayed in *esr2*, *esr3* and *esr4* mutants and wild type plants. *Pst* DC3000 is the causal organism of bacterial speck of tomato and bacterial growth is usually limited to the locally infected leaf (Agrios, 1997). *Pst* DC3000 is virulent on the *Arabidopsis* ecotype Col-0 (Whalen *et al.*, 1991) and induced resistance to this bacterial pathogen is associated with SA accumulation and *PR-1* expression (Cameron *et al.*, 1999). *Peronospora parasitica* is an oomycete biotroph and causes downy mildew in *Cruciferae* (Agrios, 1997). Many *P.parasitica* isolates have been uncovered with varying degrees of virulence on *Arabidopsis* ecotypes (Holub *et al.*, 1994). *P.parasitica* Noco2 is virulent on the ecotype Col-0 (Parker *et al.*,1993) and thus was chosen for the disease resistance analysis. Induced resistance to *P.parasitica* Noco2 in Col-0 plants is also associated with SA accumulation and *PR-1* expression (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998). It has also been suggested that ET signalling may play a role in *P.parasitica* induced resistance in Col-0 (Bowling *et al.*, 1997).

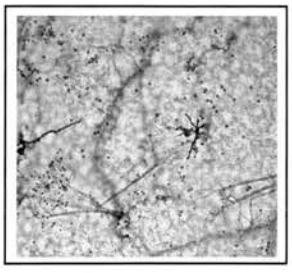
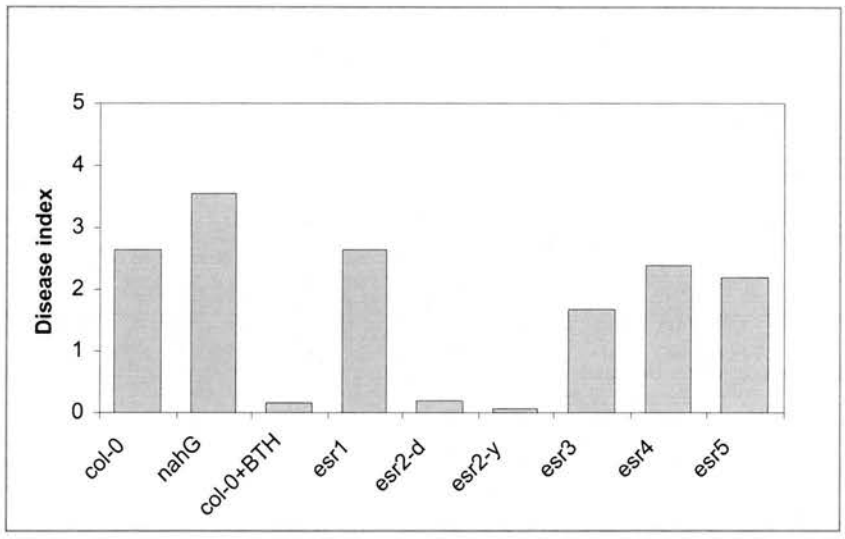


Figure 5.8. Growth of *P.parasitica* on *nahG* plants. Trypan blue staining was used to reveal development of conidiophores 10 days post-inoculation.

P. parasitica Noco2 assay

Four-week old plants were spray-inoculated with a *P.parasitica* Noco2 conidiospore suspension. The plants were maintained in a humid environment and the amount of fungal growth on leaves was visually assessed 10 days after inoculation. Col-0 plants typically showed downy mildew symptoms. *NahG* plants were previously found to be more susceptible to *P.parasitica* infection than wild type plants (Delaney *et al.*, 1994). Fig 5.8 shows a leaf from a *nahG* plant included in the assay displaying extensive conidiophore growth. Using a scoring system that ranks disease symptoms according to the number of leaves and the leaf area covered by *P.parasitica* (Epple *et al.*, 1997), the *esr* mutants were found to exhibit variation in the severity of the symptoms, from slightly more resistant than the Col-0 wild type plants to a level of resistance similar to BTH-sprayed controls (Fig 5.9A). Fungal growth was virtually completely restricted in both *esr2-dwarf* and *esr2-yellow*, whereas *esr3* and *esr5* showed a significant reduction in conidiophore development relative to Col-0. Infection was repeated twice with similar results. It was anticipated, based on the lack of constitutive *PR-1* expression in *esr1* and *esr4*, that they would not show enhanced disease resistance. The results of the *P.parasitica* assay confirmed this prediction, as growth of *P.parasitica* in both mutants was similar to Col-0 wild type levels (Fig 5.9A). Although the graph representing the spore count on 2-week old infected plants (Fig 5.9B) shows a similar overall pattern to the disease index (Fig. 5.9A), resistance observed in seedlings was reduced compared to resistance observed in adult plants (compare to BTH sprayed control). A t-test using the data obtained from the spore count indicates that *esr5* is not significantly different from the wild type control (0.33, $p<0.1$). Yet, a weak level of resistance was reproducibly observed for this mutant in several experiments. It is probably necessary to increase the number of replicates to obtain a statistically significant difference between the *esr5* plants and the wild type plants.

A



B

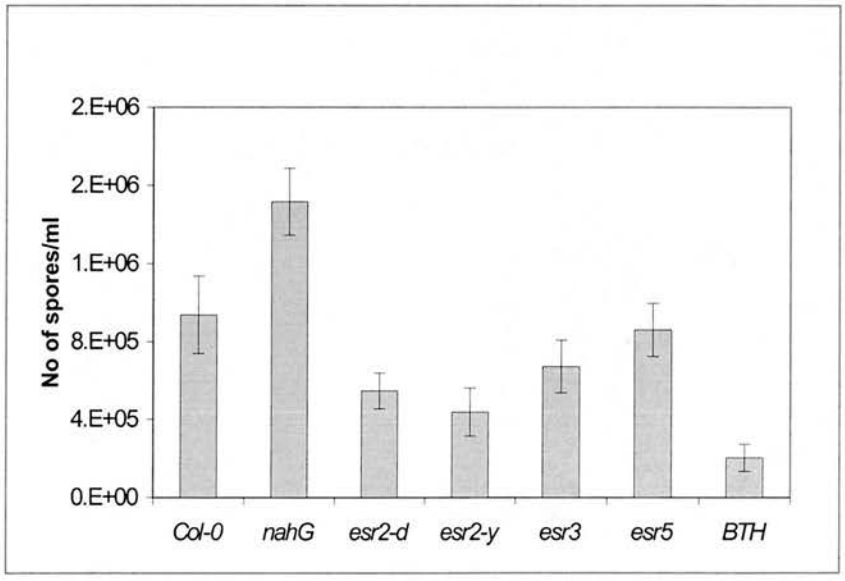


Figure 5.9. Resistance of *esr* mutants to *P.parasitica*

A)Disease index of *P.parasitica* Noco2 infection. Four-week old plants were scored at 10 days after inoculation using a scoring system developed by Cao *et al.*, 1997. Mutant and control plants were intermingled in the same tray to minimise variability in the inoculum and growth conditions. The experiment was repeated twice with similar results.
B) Spore-count assay on 2-week old plants. The spore-count on infected leaves was performed as described in materials and methods.

Pst DC3000 assay

Four-week old plants were inoculated with *Pst* DC3000 and the development of symptoms was monitored daily. Samples were collected when watersoaked patches and chlorosis of inoculated leaves became evident in Col-0 wild type plants. This occurred at three days post-inoculation. To quantify bacterial growth in *esr* mutant lines, leaf disks were produced from each inoculated leaf, homogenised, and different dilutions were plated out on KB media. After three days, the number of bacterial colonies was recorded as an indication of the bacterial titre in the different plants. Fig 5.10 outlines the results obtained for *esr3*, *esr5* and control plants. It was not possible to test *esr2* due to the difficulty of infiltrating small leaves. The difference in *Pst* DC3000 bacterial titre between wild-type plants and BTH-treated plants was not as pronounced as the difference in fungal growth observed in the *P.parasitica* assay. However, a statistically significant reduction in bacterial titre was found in *esr3* and *esr5* mutants when compared to Col-0 wild type plants.

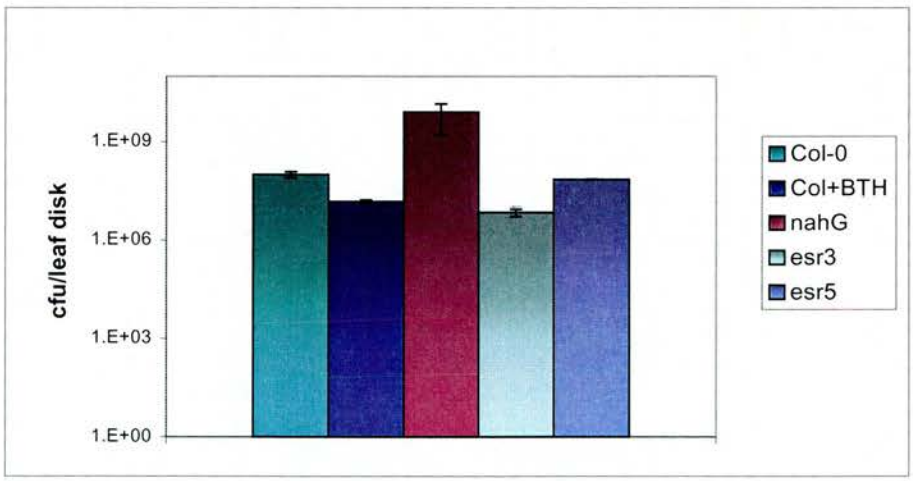


Figure 5.10. Growth of *Pst* DC3000 in *esr* mutants. Each bar represents the average value obtained from three plants. Error bars represent 95% confidence limit of log-transformed values. This experiment was repeated twice with similar results.

5.4.4 Morphological phenotype

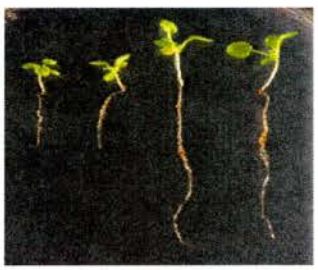
All *esr* mutants displayed visible morphological perturbations. A general reduction in plant size was observed in *esr2*, *esr3* and *esr5* mutants. This reduction was only noticeable in *esr5*, but gradually became more severe in *esr3* and *esr2*. The level of *PR-1* expression in the three mutants follows the same pattern; i.e. the amount of *PR-1* gene expression increased gradually in these three mutant lines. Thus, it is possible to correlate the degree of stunting to the level of *PR-1* expression.

esr2

Four distinct phenotypes could be related to *esr2* (Fig 5.11A). Among these, the three phenotypic classes showing constitutive *PR-1* expression (*esr2-y, esr2-d and esr2-l*)(Fig 5.5B) were considerably smaller than wild-type plants. In addition to reduced stature, *esr2-y* characteristically exhibited bleached leaves, giving the plant a yellow appearance, and spontaneous development of lesions (Fig 5.11A-Top left). Lesion formation in *esr2-y* was found to be dependent on photoperiod, as plants grown in long-day conditions showed exacerbated development of lesions. Moreover, roots of *esr2-y* seedlings were found to be shorter than those of wild type plants at the same stage (Fig 5.12). The *esr2-l* phenotypic class retained the green colour but was also smaller than wild type plants and developed lesions on leaves (Fig 5.11A-Top right). *esr2-d* was dramatically stunted and the cotyledons and primary leaves showed premature senescence (Fig 5.11A-Below left). Trypan blue staining was used to investigate the extent of cell death in leaves of *esr2* mutants (Fig 5.13). Large areas of cell death that corresponded to the sites where macrolesions were visible in leaves of *esr2-y* plants were observed (Fig 5.13B). Formation of microlesions was also visible in *esr2-d* leaves as scattered dark blue cells (Fig 5.13A).

Figure 5.11. Morphological phenotypes of *esr* mutants. Plants were photographed when 3-weeks old. A) Four distinct phenotypic classes related to *esr2* B) *esr3* plants C) *esr5* plants D) wild-type plants.

Figure 5.12. Comparison of root length in *esr2-y* (two seedlings on the left) and wild-type plants (two seedlings on the right).



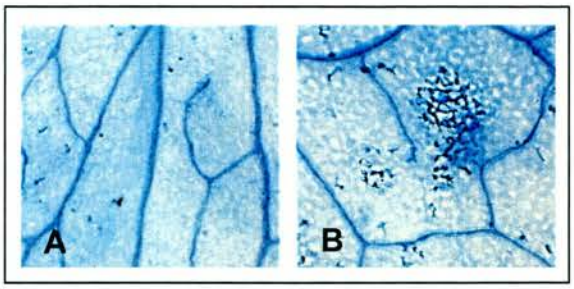


Figure 5.13. Typical lesion-formation in leaves of *esr2-d* and *esr-2-y* mutants. A leaf from 3-week old plants was stained with trypan blue to reveal dead cells. A) Micro-lesions were observed as scattered dead cells in an *esr2-d* leaf B) Macro-lesions were observed as areas of dead cells in an *esr2-y* leaf.

ET has been proposed to control the development of lesions in response to specific bacterial pathogens (Bent *et al.*, 1992; Hoffman *et al.*, 1999). As *esr2-y* showed spontaneous lesion development, we first tested the involvement of ET signalling by measuring the amount of ET produced by *esr2-y* plants. A 74% increase in ET levels was detected in *esr2-y* relative to wild-type Col-0 plants (Fig 5.14).

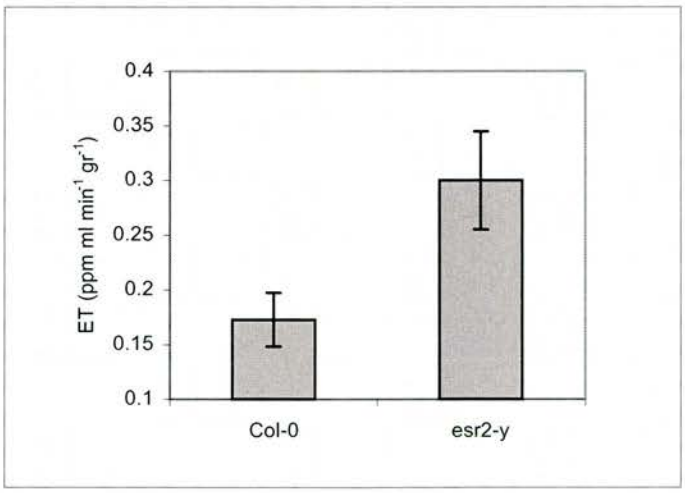


Figure 5.14. Ethylene produced by wild-type Col-0 and *esr2-y* mutant plants. Samples were taken from 3-week old plants grown on soil.

esr3

Line *esr3* displayed a combination of altered morphological and developmental characters including overall reduction in plant size, dark green and curly leaves (Fig 5.11B), increased size of flowers and siliques, delayed flowering and delayed senescence. The number of days to bolting in *esr3* plants and wild type plants was monitored under long-day conditions. Bolting was defined as the point when the stem was approximately 2cm in length. The number of bolts and bolt height of each plant were recorded at 8 weeks (Table 5.3). While both the bolt number and height in *esr3* were not significantly different from wild-type plants, the *esr3* plants were found to bolt on average, 11 days later than wild-type plants.

	Col-0	***esr3***
Days to bolting	31	42
Number of bolts (Day 57)	4 (0.7)	4.2 (0.45)
Bolt height (Day 57)	29.23 (1.84)	33.69 (2.76)

Table 5.3. Growth features of *esr3* relative to Col-0 plants.
Measurements of bolt height are shown in centimetres (cm), with the standard error (95% confidence level) shown in parentheses. Ten plants of both *esr3* and Col-0 were analysed.

esr5

The *esr5* mutant only differs from wild type plants in having slightly smaller stature (Fig 5.11C). Formation of macro or micro lesions was not observed in this mutant (data not shown).

5.5 Genetic analysis

Preliminary analysis of LUC activity segregation in T_2 plants suggested that the *esr5* mutation was dominant ($p>0.1$), while the *esr3* mutation was recessive ($p>0.1$). Segregation of *esr2* plants did not fit the expected ratio for either dominant or recessive traits (Table 5.2). Backcrosses were performed between the *esr* mutants and the parental homozygous *PR1::LUC* line to define the character of the mutations and to determine if the mutant phenotype was inherited as a single Mendelian locus. For each backcrossed *esr* mutant, at least 25 F_2 plants were imaged for LUC activity.

Initially, the T_3 progeny from the *esr2* phenotypic classes was examined to find out whether the different phenotypes within this line segregated as independent mutations (Table 5.4). The selfed *esr2-d* plants gave 100% dwarf plants in the T_3 generation suggesting that this phenotypic class defines a homozygous mutation. In addition, the T_3 progeny of *esr2-y* segregated exclusively as *esr2-y* and *wt-l* plants (Table 5.4). From these results, *esr2-d* and *esr2-y* may define two mutations that segregate independently. Backcrosses of *esr2-d* and *esr2-y* to the parental line gave F_1 progeny with no LUC activity, and F_2 populations that segregated 1:3 for constitutive LUC activity and the associated phenotype. According to the F_2 segregation of the mutant phenotype in the backcross, two single recessive mutations could be responsible for the *esr2-yellow* and *esr2-dwarf* phenotypes (Table 5.5). Allelism tests will be necessary to confirm this result. These two mutants were thus provisionally renamed *esr2* and *esr6*, respectively.

SELFED T2 PARENT		T3 Phenotypic class			
		Dwarf	Yellow	Lesions	wt-like
Dwarf	1	29	0	0	0
	2	5	0	0	0
Yellow	1	0	7	0	37
	2	0	7	0	36
Lesions	1	0	2	6	0
	2	5	6	0	0
wt-like	1	5	9	2	8
	2	7	7	3	3

Table 5.4. Segregation analysis of the *esr2* phenotypic classes. The progeny of two different T_2 lines from each phenotypic class was examined for segregating phenotypes.

When *esr3* was backcrossed to the *PR1::LUC* line, a segregation ratio of 1:1 (mutant:wild type) was obtained for the F_2 generation. From the 48 F_2 plants tested, 24 plants that had the mutant morphological phenotype also showed constitutive LUC activity, indicating that the *esr3* mutation does not segregate as a single locus ($p<0.1$) (Table 5.5). The expected ratio for the co-segregation of two loci is 9:7 (mutant:wild type), assuming that both mutations are heterozygous and dominant. This may be the case for *esr3* but a larger population of F_2 plants from independent crosses must be tested before this assumption can be proved correct.

LINE	LUC activity segregation (exp:no exp)	χ^2 (1:3)	χ^2 (3:1)
esr2-d x *PR1::LUC*	14:34	<u>0.5</u>	2.2E-13
esr2-y x *PR1::LUC*	5:19	<u>0.64</u>	8.9E-10
esr3 x *PR1::LUC*	24:24	6.3E-05	6.3E-05
esr5 x *PR1::LUC*	30:10	2.8E-13	<u>1.0</u>

Table 5.5. Segregation of F_2 progeny from backcrosses of *esr* mutants. Significant χ^2 probability values are underlined ($P< 0.1$ rejects hypothesis).

5.6 Epistasis analysis

To place the *esr* mutations in the SAR signalling pathway and investigate their dependence on SA, JA and ET signalling, crosses were performed between *esr* mutants and other characterised mutants including *nahG*, *coi1*, *etr1*, *npr1*, *eds1* and *ndr1*.

Initially, Northern analysis was used to determine whether constitutive *PR-1* gene expression was affected in *esr2*x*nahG*, *esr2*x*coi1* and *esr2*x*etr1* double mutants. The *esr2*x*nahG* double mutant was phenotypically distinct from both *esr2* and *nahG* parental lines. It resembled *esr2* in size but lacked the characteristic yellow colour and development of lesions. In contrast, the *esr2* phenotype was not discernible in the *esr2*x*coi1* and *esr2*x*etr1* double mutants. Preliminary results from Northern blot analysis showed that *PR-1* expression was partially suppressed in *esr2*x*nahG* and surprisingly, it was totally suppressed in *esr2*x*coi1* and *esr2*x*etr1* (Fig 5.15).

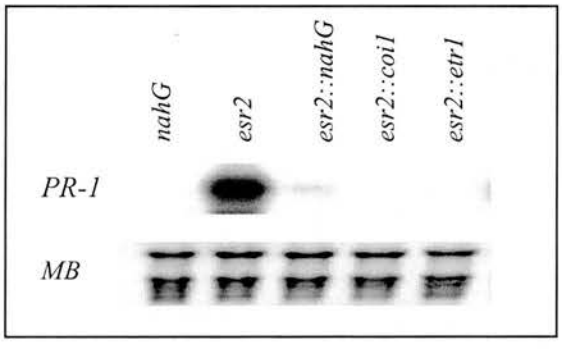


Figure 5.15. Effects of *nahG*, *coi1* and *etr1* on *PR-1* expression in the *esr2* mutant. Total RNA was extracted from 3-week old plants. Methylene blue staining was used to visualise loading.

CHAPTER 6

6.1 Discussion

To isolate *Arabidopsis* mutants involved in the SAR signal transduction network, an activation-tagged population was screened using a designer genetic background. The transgenic line transformed with the activation tagging vector pSKI015, contained the SA-responsive *PR-1* promoter fused to the firefly *LUC* gene. *LUC* is a non-destructive and non-invasive reporter gene. This opened a number of possibilities, such as following *PR-1* gene expression in real time and analysing *PR-1* gene induction in response to SA and its functional analogue, BTH. Constitutive *PR-1* candidate mutants were thus identified by constitutive LUC activity controlled by the *PR-1* promoter. Suppressor candidate mutants were identified by loss of LUC activity following induction by BTH. A set of 121 suppressor candidate mutants were isolated, showing LUC activity in no more than ~ 25% of imaged T_2 seedlings. While it cannot be ruled out that the majority of these putative mutants constitute activated or knocked-out genes, because of time limitations, an extensive characterisation of such a large group of putative suppressor mutants was unfeasible. Therefore, our work focused on the analysis of the constitutive *PR-1* candidate mutants. Among the five putative mutants isolated in the screen for constitutive LUC activity, four were found to be resistant to the virulent oomycete pathogen *P.parasitica* Noco2 and showed constitutive expression of the endogenous *PR-1* gene. Therefore, these four mutants, named *esr2*, *esr3, esr5* and *esr6* for *e*nhanced *s*ystemic *r*esistance, proved to have altered control of SAR. Although SA levels were not determined in the *esr* mutants, it is likely that constitutive *PR-1* expression is the result of increased SA levels in at least some of these mutants.

Based on the genetic analysis of the *esr2* phenotypes segregating within this line, two different recessive mutations could be identified. The mutation associated with spontaneous development of lesions and yellowing was designated *esr2* and the mutation associated with dwarfing and early senescence was designated *esr6*. Both

esr2 and *esr6* displayed levels of resistance to *P.parasitica* Noco2 comparable to BTH-induced plants. In addition, the amount of ET evolved by *esr2* plants was found to be significantly greater than wild-type plants. To further investigate the role of ET signalling in *esr2* and define where the *esr2* mutation functions in the SAR signalling network, we began by analysing F_2 plants from crosses between *esr2* and *nahG*, *coi1* and *etr1*. Surprisingly, the *esr2* cell death phenotype was suppressed in all three crosses. *PR-1* expression was strongly reduced in the *esr2*x*nahG* double mutant and completely abolished in both *esr2*x*coi1* and *esr2*x*etr1* double mutants. The *esr2*x*nahG* plants were also smaller than wild-type plants indicating that removal of SA blocked cell death but only partially suppressed the reduced stature of *esr2*. Consequently, other defence pathways besides the SA pathway must be activated in *esr2*x*nahG*. The absence of lesions, reduced stature or yellowing in the *esr2*x*etr1* and *esr2*x*coi1* double mutants further support the idea that blocked ET/JA signalling can suppress constitutive *PR-1* expression and the co-segregating phenotype in *esr2* plants. In conclusion, cell death in *esr2* is strictly dependent on both SA and JA/ET signalling, however, *PR-1* expression is only partially dependent on SA signalling but totally dependent on JA/ET signalling. These findings are difficult to reconcile with the idea that regulation of *PR-1* expression and cell death occurs through only the SA pathway (Delaney *et al.*, 1994; Ryals *et al.*, 1996; Dangl *et al.*,1996). The lack of a central role for SA in response to avirulent or virulent pathogen infection is unusual in the literature but not without precedent. In *Arabidopsis*, *RPP7*-mediated resistance to *P.parasitica* as well as *RPM1*-mediated resistance against *Pst* was shown to be independent of SA (McDowell *et al.*, 2000). The tomato *Cf-2* and *Cf-9* resistant response to *C.fulvum* and the resistant response to *Xanthomonas campestris* pv. *vesicatoria* are also independent of SA (Dixon *et al.*, 1996; Jones *et al.*, 1994; Hammond-Kosack and Jones, 1997). Additionally, the *Arabidopsis* lesion mimic mutant *acd5* accumulates SA but does not induce SAR and shows susceptibility to infection by *P.syringae* (Greenberg *et al.*, 2000). Similarly, *dth9* is more susceptible to bacterial and oomycete pathogens and shows a compromised SAR response that cannot be complemented by SA (Mayda *et al.*, 2000). Furthermore, an SA-independent mechanism of cell death was proposed to induce HR in *nahG* plants treated with ozone, and analysis of the *acd6* mutant showed that cell death is a

consequence of SA interacting with a second, unidentified signal (Rao *et al.*, 2000; Rate *et al.*, 1999). Therefore, it appears that both SA-dependent and independent mechanisms can result in HR and disease resistance. The roles of ET and JA are equally controversial. ET insensitivity has been correlated with reduced symptoms (Lund *et al.*, 1998; Hoffman *et al.*, 1999), increased symptoms (Knoester *et al.*, 1998) or no effect in disease outcome (Hoffman *et al.*, 1999). Yet, alterations in ET synthesis or perception are known to greatly influence accumulation of SA in infected tissues (O'Donnell *et al.*, 1996). In this context, over-expression of the ET-responsive factors Pti4, Pti5 and Pti6 in *Arabidopsis* caused constitutive expression of the SA-regulated genes *PR-1* and *PR-2* (Gu *et al.*, unpublished results). It has also been shown that ethylene regulates JA biosynthesis during a wound response (Kubigsteltig *et al.*, 1999). Moreover, it has been demonstrated that resistance against *Psm*ES4326 in *cpr6*, and against *Pst* and *Peronospora* spp. in *ssi2* can be mediated *via* an NPR1-independent pathway that requires both ET/JA and SA signalling components (Clarke *et al.*, 2000; Shah *et al.*, 2001). Thus, there may be pathogen-related specific functions for the different components of the SA, ET and JA signal transduction pathways (Thomma *et al.*, 2001). The *esr2* mutation may define a gene encoding a key regulator of this novel defence signalling pathway. The enhanced resistance against *P.parasitica* observed in *esr2* might be correlated to the expression of a specific set of *PR* genes. Further RNA gel blot analysis of crosses between *esr2* and *nahG*, *etr1*, *coi1* and other SAR mutants will show if *PR-1* is co-expressed with other *PR*-genes and whether expression of these genes is affected by known regulatory components of the SA, JA and ET signalling network. To test the requirement of SA, ET and JA for induction of resistance in *esr2*, pathogenicity assays will be carried out in F_2 plants from the *esr2*x*nahG*, *esr2*x*etr1* and *esr2*x*coi1* double mutants. It will also be important to investigate the specific role of SA, and JA/ET signalling in resistance against different virulent bacterial and fungal isolates. In this context, the possibility that *esr2*x*nahG* plants may retain resistance to pathogens through activation of JA/ET responses is of particular interest. The identification of the *esr2* mutant lends support to the notion that ET/JA signalling plays an important role in resistance to pathogens and will possibly provide new

information on the complex signalling network that controls disease resistance. A similar analysis of the *esr6* mutant will surely yield interesting results.

PR-1 expression in *esr3* seems to follow a developmental pattern rather than constitutive activity *per se*, suggesting that developmental changes affect the function of ESR3. This hypothesis is supported by the observation that in *esr3,* LUC activity increases continuously during the vegetative growth stage. However, the diverse morphological and developmental perturbations observed in *esr3* could result from a second, tightly linked mutation as suggested by the segregation analysis of the backcrosses. An alternative and perhaps more interesting interpretation is that *ESR3* co-regulates defence and developmental pathways. A striking feature of *esr3* is the delayed flowering and senescence. Although plant senescence is a developmentally programmed event, the initiation and progression of senescence can be influenced by a number of hormones, such as ABA, MeJA and ET (Grbic and Bleecker, 1995; Oh *et al.*, 1997). These plant hormones have diverse effects on leaf senescence implying that mutations causing defects in the JA/ET signalling pathways underlying the perception and response to these hormones can affect the normal progression of senescence. Consistent with this idea is the observation that ethylene-insensitive *etr1* and *ein2* mutants show delayed senescence (Mach *et al.,* 2001). The observed delay of leaf senescence in *esr3* may be attributed to mutations affecting JA/ET signalling pathways that are common to both defence and senescence. It will be interesting to investigate how the *esr6* and *esr3* mutations can respectively promote or delay this process. A genetic analysis of crosses to mutants involved in JA/ET signalling as well as mutants involved in other pathways controlling growth, longevity and senescence may elucidate how defence relates to development in plants. In this context, examination of the cell structure and organisation may reveal further alterations. Another interesting question raised by the analysis of *esr3* is whether the constitutive expression of *PR-1* and possibly other defence-related genes in the absence of cell death can confer enhanced resistance to necrotrophic pathogens. It has been suggested that HR-mediated cell death can facilitate infection by necrotrophs (McDowell *et al.*, 2000; Govrin and Levine, 2000). Recent findings indicate that necrotrophic pathogens such as *B.cinerea* use oxidative reactions

analogous to the oxidative burst elicited during the HR, to attack and destroy plant tissues (Deighton *et al.*, 1999). Conceivably, elevated levels of *PR-1* and other *PR* genes without triggering cell death may interfere with the early stages of necrotroph infection.

The impact of the *esr5* mutation on *PR-1* activation is much weaker compared with that of *esr2*, *esr3* or *esr6*. This is probably reflected in the slight reduction in plant size and the modest resistance to *P.parasitica* Noco2 and *Pst*. Because *esr5* plants do not exhibit lesion development, either macro or microscopically, we speculate that the *esr5* mutation is downstream of the HR. Further investigation of crosses to other SAR mutants will help to understand the function of *esr5* in disease resistance.

The number of T-DNA insertions in *esr* mutants was determined by Southern blot analysis using probes for the CaMV 35S enhancer and a pBluescriptSK region within the activation tagging vector. However, the hibridisation experiment using a Bluescript probe showed several bands below the predicted size range and therefore the results were unconclusive. The *esr3* mutant contained three copies and the *esr5* mutant contained two copies as observed in the hibridisation with the CaMV 35S probe. For the *esr2* mutant, no hybridising bands were detected using the CaMV 35S enhancer probe but a weak band in the expected size range (>5 kb) was detected with the Bluescript probe. If this result is confirmed it would suggest the vector was deleted or rearranged following transfer into the plant genome. It remains to be investigated whether any of these mutants is associated with a T-DNA insertion. If a link between one of the mutant phenotypes and the insertion is confirmed, the vector sequence flanking the mutated gene can be used as a tag for gene cloning. Although the *esr3* phenotype appears to be segregating with BASTA resistance and is thus linked to a T-DNA insertion, the genetic analysis indicates that more than one locus contributes to the mutant phenotype. Hence, linkage to a specific T-DNA insertion remains to be established. In the case of *esr5*, the mutant phenotype was found to segregate independently of BASTA resistance despite the presence of two T-DNA copies. This implies that a map-based cloning approach will be necessary if identification of the *ESR5* gene is to be pursued.

The system described in this study was well suited for a large-scale screen. However, the rate of untagged mutants is high (3/5 in 5000 lines screened). The reason for the low tagging rate might be intrinsical to the activation tagging system. It has been often reported that mutations arising from insertional mutagenesis are complex, characterised by multiple, inverted or tandem copies and rearranged T-DNA, making molecular analysis difficult (Tissier *et al.*, 1999). Our results indicate that vector rearrangements in activation tagged lines may be more common than previously documented and therefore, a screen on a larger number of activation tagged lines is required to isolate gain-of-function mutants that exhibit straightforward genetic behaviour. Alternatively, a screen on T_1 mutant lines will unequivocally result in the identification of co-dominant and possibly tagged mutations.

6.2 Significance of the research and potential impact in agriculture

It is becoming increasingly apparent that JA/ET signalling has a major role in resistance against microbial pathogens (Thomma *et al.*, 2001). Our results underline the importance of JA/ET for the establishment of resistance *via* activation of distinct signalling pathways. Gene expression profiles of *Arabidopsis* have grouped defence genes in three categories: 1) induced by SA but repressed by JA/ET; 2) induced by JA/ET but repressed by SA and 3) genes whose induction requires SA and components of both JA and ET signalling pathways (Schenk *et al.*, 2000; Glazebrook, personal communication). Induction of *AtPRB1* by MeJA and ET, and inhibition by SA indicated that this novel basic PR-1 is encoded by a gene that belongs to the category that is regulated exclusively through the JA/ET pathway. These genes are possibly involved in resistance against necrotrophic pathogens. In contrast, analysis of the *esr2* mutant isolated in our screen indicated that expression of the acidic *PR-1* is dependent on SA, JA and ET accumulation. Analysis of the *esr2*x*npr1* double mutant will be crucial to establish if *esr2* is integral to the NPR1-independent pathway uncovered by studies of the *cpr6* mutant (Clarke *et al.*, 1998). Clarke *et al.* demonstrated that activation of a SA-dependent but NPR1-independent

pathway that requires JA/ET leads to *PR-1* expression and resistance to *P.parasitica* Noco2. Significantly, the observation that in *esr2*, both SA accumulation and JA/ET signalling are essential for *PR-1* expression suggests that ESR2 may act as a regulator of this unexplored signalling pathway.

A promising approach to develop disease-resistant crops is to exploit these naturally occurring defence mechanisms. Over-expression of key regulators such as NPR1 and NDR-1 in *Arabidopsis* (Cao *et al.*, 1998; Repetti and Staskawicz, 1998) and Prf in tomato (Oldroyd and Staskawicz, 1998) have validated this approach. While over-expression of these genes can effectively induce SA-dependent responses that result in resistance to a number of pathogens, a regulator that induces both SA and JA/ET responses is likely to produce broader resistance to both biotrophic and necrotrophic pathogens. From a biotechnological perspective, the cloning of *ESR2* may open new opportunities to engineer resistance derived from the activation of one/several signalling pathways. Nevertheless, it will be necessary to fully understand the function of ESR genes before they can be considered for use in crop protection. Future work will focus on the genetic analysis of double and triple mutants that combine *esr* mutations with mutations that disrupt signalling at different points of the SA pathway, including *npr1*, *eds1* and *ndr1*. One of the most useful tools for the characterisation of disease resistance signalling pathways and the subsequent seeking of effective means for plant disease control is microarray technology. Gene expression patterns of resistant and susceptible plants, mutants, or transgenic plants, with or without pathogen inoculation, can be compared to identify genes involved in resistance (Maleck *et al.*, 2000; Schenk *et al.*, 2000). The transcription profiles of *esr* single and double mutants will lead to the identification of regulatory genes required for *esr*-conferred resistance as well as genes that are co-regulated by SA, JA and ET pathway activation. Furthermore, it will be possible to determine the specific *PR* gene combinations necessary for resistance to a particular pathogen. Microarrays will be used in combination with reverse genetics for characterisation of the underlying regulatory pathway.

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